SOIL BIOLOGY

P. Nannipieri · K. Smalla (Eds.)

Nucleic Acids and Proteins in Soil



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Preface

This book is devoted to nucleic acids and proteins in soil. The intention is to compile our present knowledge and understanding of the importance of nucleic acids and proteins present in soil either inside or outside of microbial cells. Since microbe-driven functions are key for an enormous range of nutrient cycling and geochemical processes in soil it is expected that a better understanding of the structural and functional diversity and dynamics of the microbial community will enable us to understand and eventually predict or manage soil functions of importance. This is the reason why there are so many chapters on soil microbial community analysis in a book entitled "Nucleic acids and proteins in soil". The rather ambitious idea behind the book was to link traditional soil microbiology, biochemistry and chemistry approaches with the more recently developed ones in the field of molecular microbial ecology, all aiming at a better understanding of soil functioning. Whereas only a few studies on the analysis of nucleic acids in soil had been carried out before the 1990s, the bibliography on the extraction of enzymes, interactions between proteins and soil colloids and quantification of protein N in soil was at that time already rather extensive.

The first chapter by Stres and Tiedje is entitled "The new frontiers in soil microbiology: how to link structure and function of microbial communities?". Stres and Tiedje consider the attempt to link microbial community structure and function in a predictive way as a "Decadal Grand Challenge" in particular because microbial communities in soils are very diverse and complex, and vary at very small scales. Several chapters of the book deal with recent methodological advances made to uncover the links between structure and functions of soil microbial diversity. The basis of most cultivation-independent approaches is the extraction of either nucleic acids or proteins directly from soil. In Chaps. 3 and 11, written by Bakken and Frostegård and by Wellington et al., the reader will learn more about the ongoing challenge to obtain nucleic acids from the "dirt" which are suitable for molecular analysis. The different steps required for nucleic acid extraction are shown, secrets and useful tricks are mentioned, and the various potential biases are critically discussed. In Chap. 3, Bakken and Frostegård cite a line from one of Leonard Cohen's songs, "... there is a crack in everything, that's how the light comes in"; a biased method is problem-

atic (cracks) until we know the extent and the reasons for the bias. With that knowledge, experiments can be designed to exploit the bias to unravel new phenomena ("... how the light comes in"), and this rather philosophical consideration seems an appropriate approach for many methods discussed in this book. The molecular analysis of nucleic acids extracted from soils allows us conversation with the silent majority of soil microbes. While the analysis of polymerase chain reaction (PCR)-amplified rRNA genes from TC-NA by community fingerprinting techniques or cloning and sequencing (Chaps. 8 and 11) is nowadays a standard technique, the use of gene arrays for structural and functional analysis of soil microbial communities still remains a huge methodological challenge (see Chap. 9). When these techniques are applied for TC-NA analysis an idea of the kinds of numerically dominant microbial populations and genes present is obtained. However, in general, these approaches give us relatively little idea about the in situ metabolic functions. One powerful tool for microbial ecologists providing a culture-independent means of identifying microbial populations conducting certain microbial processes is stable isotope probing (SIP). The potentials and limitations of SIP are addressed in Chap. 10 by Manefield et al. In recent years, the detection and quantification of gene expression in soil has become feasible, as illustrated in Chap. 11 by Wellington et al. The obvious next step is the analysis of the soil proteome, a subject discussed in the chapters by Ogunseitan and Nannipieri (Chaps. 5 and 4). Although this methodological approach is still in its infancy it can certainly make use of the long-term experience in analysing proteins in soil.

Only recently has the full extent to which horizontal gene transfer processes drive bacterial diversity and adaptability become evident. Three chapters of this book are devoted to this issue. How to assess the abundance and diversity of mobile genetic elements (MGE) in soil bacterial communities? Methods presently used, their potentials and limitations are summarised in the chapter by Smalla and Heuer (Chap. 13). A comprehensive overview of our present knowledge on gene transfer by conjugation in soil and the various biotic and abiotic factors influencing frequencies and establishment is given in Chap. 14 by Van Elsas et al. Chap. 15 on transformation by Mercier et al. makes the link between free DNA in soil and bacteria with a particular emphasis on the transformation of bacteria by transgenic or transplastomic DNA. As discussed by Nielsen et al. in Chap. 7, the interactions of nucleic acids and proteins with soil colloids protect these molecules against enzymatic degradation. Indeed, an intriguing aspect of soil as a biological system is the capacity of soil colloids to adsorb or entrap important molecules such as nucleic acids and proteins. The origin of DNA in soil and the factors influencing the persistence are addressed by Wackernagel in Chap. 6. Nucleic acids adsorbed or bound by clays are protected against degradation by nucleases. However, they can

be taken up by competent bacterial cells and can be integrated into the recipients' genome by homologous recombination or homology-facilitated illegitimate recombination. A less microbe-focused and a broad view on state, location and function of enzymes in soil is given by Gianfreda and Ruggiero (Chap. 12).

It is amazing that on average only 4% of the total N in soil is present in microbial biomass N whereas a high proportion (from 30–50%) is present as amino acids after acid hydrolysis. Thus it is reasonable to hypothesise that the amount of extracellular protein N is also considerable and it is much higher than the amount of intracellular protein N in soil (see Chaps. 2 and 4, respectively). Indeed, as discussed by Kögel-Knabner in Chap. 2, the use of NMR has confirmed that extracellular protein N is present in soil.

The final three chapters of this book (Chaps. 16–18) concentrate on marker and reporter genes and their use in soil microbial ecology. Although these techniques rely on introduced microbes equipped with respective marker and reporter genes, they allow researchers not only to follow the fate and activity of introduced microbes, but also to discover how they respond to environmental triggers. Thus these techniques considerably contribute to a better understanding of the microenvironment surrounding microorganisms inhabiting soil and to locally quantify the activity of stimulators, inhibitors and pollutants with a better link between soil microbiology and soil chemistry and biochemistry.

Generally, the information obtained by using molecular techniques are considered as part of 'modern' soil microbiology and biochemistry, whereas the information retrieved by classical techniques, such as soil respiration, microbial biomass, enzyme activities, etc., are considered as black boxes, sum parameters and rough estimates. Perhaps for this reason there is a tendency to separate the two approaches which is conceptually wrong. In this book we have tried to overcome this separation because, in our opinion, both approaches should be more often combined to gain better insight into the microbial processes occurring in soil. The use of stable isotope probing (see Chap. 10) is one of the most fruitful combinations of the two approaches.

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1 New Frontiers in Soil Microbiology: How To Link Structure and Function of Microbial Communities?

Blaž Stres, James M. Tiedje

1.1 Introduction: A Framework for the Structure–Function Grand Challenge

The great challenge in microbial ecology of the inaugural decade of the twenty-first century is to resolve and then understand the linkage between microbial community structure and its function. This goal is based on the fact that the primary value of microbes is their function and the belief that understanding the composition, relative abundance, biochemical diversity and dynamics of the microbial community will help us understand and eventually predict or manage function in microbial communities of importance. However, microbial communities are very diverse and complex, and vary at very small scales and in which cells are active. Furthermore, measurement of microbial activities at the relevant spatial and temporal scales, especially in soil, is no less challenging. Hence, linking microbial community structure and function in predictive ways must be considered the Decadal Grand Challenge!

The desire to link function to structure derives from microbial diversity, i.e. that microbes vary enormously in their biochemical potential. This is understandable. They have been on earth for 3.8 billion years and have exploited most energy niches and adapted to a tremendous range of conditions. Their contemporary progeny carry the genetic heritage from that extensive diversification and selection. The extant diversification represents a continuum from a range of major features that we first think about as functional diversity such as differences in energy-producing redox couples, metabolic pathways, morphological adaptations (e.g. spores, filaments, motility, surface-to-volume ratio), and tolerance to environmental stresses such as desiccation, pH and temperature extremes, salt, and growth kinetics (r and K selected populations). However, diversification has also

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produced a number of other variants that can have major effects on function but may be more difficult to recognise in community structure studies such as differences in enzyme kinetics (e.g. K_m, V_{max}, K_i), gene regulation (signal transduction, regulatory networks, response time, specificity), enzyme stability/turnover, rate and mechanisms of genetic change, and gene redundancy in the same organism (orthologues, paralogues, isozymes). The combination of these traits in members of the populations can create large differences in functional diversity, e.g. low K_m, psychroactive, with rapid induction response populations, versus others that vary in these and other traits. When certain traits of diversity can be associated with a particular condition, usually one offering a competitive advantage, we then have a useful linkage of community structure and function.

Given the small size of the organisms and the tendency to attach to surfaces, individual microbial cells interact with one another only over short distances controlled by diffusional processes and gradients. Direct competition among microorganisms in soil is limited by spatial isolation and resource heterogeneity among various habitats thus resulting in communities with equally abundant and uniformly distributed bacterial species (Tiedje et al. 2001; Zhou et al. 2002; Roberts et al. 2004). A two-species test of the hypothesis that spatial isolation influences microbial diversity in soil indicates that spatial isolation, created by low moisture content, plays an important role in structuring soil microbial communities (Treves et al. 2003). Habitats such as soil should also support a large number of different species expressing significant niche overlap. Mechanistic models for the differential performance of species over time within communities (Roberts et al. 2004) and experimental data (Rainey and Travisano 1998; Riley et al. 2001; Dunbar et al. 2002) predict that over time the average niche breadth of individuals will decrease resulting in an increased proportion of specialists. The course of succession will be constrained by selective pressures of physical, chemical and biological conditions existing within a given niche. The balance between generalists and specialists is important as an unpredictable extinction of community members dominated by generalists may hamper community function to a greater extent than in a community comprised of specialists. The large number of organisms capable of performing individual biochemical reactions enhances the probability of restoring functions by replacing extinct, diminished or inactive populations (Roberts et al. 2004). However, the relationship between microbial diversity–community structure and soil processes was found to be non-linear as many processes are carried out in specialised microbial consortia. Small linear changes in microbial diversity may result in non-linear changes in processes, especially if the number of organisms drops below the number of original functional groups of organisms in a sample (Franklin et al. 2001; Griffiths et al. 2004). The redundancy of function within a community serves to dampen the loss

of ecosystem function in two ways: by providing functional redundancy for the same pathway/enzyme type or by different pathways that achieve the same processing goal. Diversity seems to contribute beneficially to redundancy by providing multiple ecotypes capable of performing an equivalent function albeit with a different community structure. Under perturbed conditions, however, significant deviations in functional traits are possible and, as the perturbation becomes more severe, the deviation more likely. In these cases numerically minor but important populations can bloom and dominate function (Hashsham et al. 2000; Roberts et al. 2004). In short, soil microbial diversity is enormous, the environmental responses and interactions are complex, but we are beginning to understand the causes for some of the simpler cases.

Analysis of spatial distribution of bacteria at microhabitat levels revealed that 80% of the bacteria in fertilised soils were located in micropores of stable soil aggregates (Ranjard and Richaume 2001) suggesting that these habitats offer the most favourable conditions for microbial growth (water and substrate availability, gas diffusion, predation protection). Nunan et al. (2002) showed that the degree of randomness in the distribution of bacteria is greater in the subsoil than in topsoil suggesting that bacteria have a hot-spot distribution in bulk soil, with the hot spots being smaller in the subsoil than in the topsoil. Sessitsch et al. (2001) showed that microbial diversity increases with decreasing particle size and that particle size has a more notable impact on microbial diversity and community structure than does bulk pH and the amount and type of organic substrate. Other investigations of different soil types found that the type and amount of available organic substrates strongly influence the abundance of microbial groups and their functional diversity in soil ecosystems (De Fede et al. 2001; Grayston et al. 2001; Smit et al. 2001). An emerging theme is that structure–function interactions and vice versa are not easily predicted as either can produce positive, neutral or negative effects on the other depending on the environmental context. In this respect, ecological responses to multiple stressors over long time scales have yet to be thoroughly explored.

1.2 Microbial Community Structure: One-Half of the Structure–Function Paradigm

Ecologists are faced with the challenge of understanding the structure and function of systems, the component parts of which interact with each other in very complex and diffuse ways at multitudes of scales in space and time. The approaches that are regularly deployed by ecologists to make sense of such complexity vary in their degree of abstraction from nature

ranging from field observations, through experimentation in the field to experiments in the laboratory. Some of them also include mathematical modeling and rigorous hypothesis testing, although these are not very common.

In recent years, the exploration of microbial biodiversity has taken a giant step forward by introduction of methodology that has enabled microbiologists to detect and sometimes partially characterise uncultured and thus previously unknown organisms. The so-called cultivation-independent methods have provided the opportunity to more comprehensively determine the composition of microbial communities and identify numerically important, but not yet cultured, microorganisms. Ironically, the use of classical approaches to characterise soil environment and processes are often neglected. Given the central role of prokaryotes in the environment, defining the extent of microbial diversity in microbial communities, as well as spatial and temporary variations in their composition and function, have now become more important to ecologists.

Development of new methods for culture-independent studies of microbial communities was spurred by reports of the vast diversity of uncultured life in natural habitats in the last two decades. Re-association of microbial community genomes has shown that the community genome size equals the size of 6,000–10,000 *E. coli* genomes in unperturbed organic soils and 300–1,500 genomes in arable or heavy-metal-polluted soil (Torsvik et al. 1998; Øvreas 2000). The total genomic diversity recovered by classical culturing methods was less than 40 genomes. A number of methods with higher resolution have been developed to characterise microbial community structure. Most of these methods are based on analyses of rRNA genes that enabled discovery of many novel phylogenetic lineages. However, the rRNA gene sequences that have revealed a remarkably vast microbial diversity generally provide few direct clues regarding the interactions and metabolic capabilities of the microorganisms that these sequences represent. Microscopy and nucleic acid characterisation, fluorescent in situ hybridisation (FISH), and especially sequencing of 16S rRNA genes, have been deployed in combination to identify unknown bacteria (Derakshani et al. 2001) using community DNA extracted from the environment. DNA reassociation in combination with guanine+cytosine (GC) content (Nüsslein and Tiedje 1998, 1999) provides broad-scale information on genetic diversity of a microbial community and detects changes in community structure. However, description of other diversity parameters such as richness, evenness, dominance (Magurran 1988) and composition is not feasible using only this approach. Fractionation of community DNA samples differing in GC content by ultracentrifugation enables coupling of this method to polymerase chain reaction (PCR)-based fingerprinting or cloning and sequencing of target genes to give a much higher resolution of community

structure and population dynamics over time (Nüsslein and Tiedje 1998, 1999; Holben et al. 2004).

Techniques producing community fingerprints such as denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), amplified ribosomal DNA restriction analysis (ARDRA), single-stranded conformation polymorphism (SSCP), amplified ribosomal intergenic spacer analysis (ARISA), amplified fragment length polymorphism (AFLP), length-heterogeneity PCR (LH-PCR), terminal-restriction fragment length polymorphism (T-RFLP) and a non-DNA-based fingerprinting technique, PLFA, provide rapid means to assess information on the species or operational taxonomic unit composition and can be used to compare distribution of common (detectable) otus present in environmental samples. The method offering the highest degree of phylogenetic resolution available to culture-independent methodologies is rRNA gene clone libraries, but this is cumbersome for analysis of large sample numbers that may be produced by studies of temporal or spatial variability of microbial communities (Braker et al. 2000; Kitts 2001). Community analysis by T-RFLP currently offers a compromise between sample throughput and phylogenetic resolution. Recent advances in T-RFLP data analyses have enabled phylogenetic placement of terminal restriction fragments (phylogenetic assignment tool; Marsh et al. 2000; Blackwood et al. 2003; Kent et al. 2003) from multiple restriction digests. The similarity of T-RFLP profiles can be assessed statistically to evaluate significance in richness or evenness of microbial communities and analysed using hierarchical clustering algorithms, principal component analysis and self-organising maps (Blackwood et al. 2003; Wolsing and Priemé 2004).

Due to rapid accumulation of 16S rRNA and other housekeeping and functional gene sequences, the need for statistical and mathematical approaches of pattern recognition to derive meaningful conclusions from large datasets has become obvious. Parametric and non-parametric approaches to estimate microbial diversities from gene sequences were developed (Chao 1987; Watwe and Gangal 1996; Hughes et al. 2001) as statistical analysis adds rigour to comparisons of diversity by demonstrating the lack of significance and enables detection of differences that are not directly apparent. Broader application of these tools is essential for meaningful conclusions about the factors that influence richness and abundance of microbial communities in their natural habitats (Morris et al. 2002).

The biases introduced by PCR amplification have long been known (cf. Kent and Triplett 2002) and so PCR-based methods do not describe total species richness. Despite the inherent problems of PCR the combination of these techniques with traditional activity measurements can reveal important insight into microbial community structure, ecology and function. Recently, targeting of recombinant sites that flank gene cassettes associated

with integrons by gene cassette PCR has enabled recovery of new genes with clear homologies to known genes indicating that mobile gene cassettes are widespread in natural environments and are likely to contribute significantly to bacterial diversity and evolution (Stokes et al. 2001; Holmes et al. 2003; Blair et al. 2004). Additionally, functional diversification within a population originating from one strain was detected in a prolonged stationary phase. Aged cultures increased in diversity in the face of extremely limiting nutrient resources and intense competition. An increase in mutation frequency was observed eventually leading to the evolution of microbial diversity (Finkel and Kolter 1999; Zinser and Kolter 1999). In this respect, ecological dynamics are ultimately inseparable from evolution, highlighting the importance of considering real-time evolution in understanding ecological processes such as population dynamics.

In summary, the understanding of soil microbial structure has progressed tremendously over the last 20 years since the introduction of molecular methods, but, due to the scale and complexity of the soil ecosystem, we may be no more than 10% of the way towards understanding structure at a level needed to understand important functions.

1.3 The Other One-Half: Functional Traits of Microbial Communities

Functional redundancy in microbial communities can most easily be observed when numerous bacterial isolates grow on diluted selective media. The potential for redundancy among microbial communities can be estimated if the approximate numbers of populations within a gram of soil are assessed (Torsvik et al. 1998). Genome sequences provide us with data on partitioning of genes among different processes thus yielding an astonishing 10^5 to 10^6 potentially unique functions that could be carried out by the community in a gram of soil (Roberts et al. 2004). In contrast, there is a large number (at least 500) of measurable functional (enzymatic) traits that are highly informative of community functional capabilities. The most commonly applied include: substrate-induced respiration (SIR), carbon and nitrogen mineralisation, dehydrogenase, chitinase and similar gross enzymatic tests such as nitrification and denitrification potential activities. Only occasionally have these and other functional traits been mapped across a span of ecological parameters. A challenge in doing so is that process regulators affect various microbial populations differently and can induce a range of responses in different microbial communities (Tiedje 1994; Conrad 1996), e.g. performance of a biochemical reaction as influenced by different temperatures, pH value, concentrations of inhibitors or activators

or the lag time of response; the differential use of certain electron donors and acceptors and as a function of concentration (starvation vs. feast); the efficiency of consumption; or the rate of production of target compounds across a span of water availability. Standard methods that enable monitoring of gross microbial processes were developed decades ago and have largely focused on measurable traits linked to biogeochemical processes of carbon, nitrogen, phosphorus and sulphur cycles (Weaver et al. 1994; Robertson et al. 1999). Measurements of products and remaining substrate concentrations enable estimation of kinetic parameters. Different kinetic parameters within a guild give different substrate turnover rates and different residual concentrations, which can be important functional differences in a community. Seemingly, communities in soils can differ significantly in their kinetic properties of particular enzymatic reactions. Whether this is due to a difference in overall K_m or V_{max} values that is a net outcome of K_m and V_{max} values of various organisms performing the reaction (the amount of enzyme and in situ substrate concentration), or simply to a population with a different K_m or V_{max} values, will remain a hard task to determine, despite the possible use of monoclonal antibodies directed toward the enzyme of choice, mRNA and functional gene analysis (Metz et al. 2003).

As an example, measurements of denitrification enzyme induction time and activity in response to pH, oxygen level, and temperature (Cavigelli and Robertson 2000, 2001; Bergsma et al. 2002; Öquist et al. 2004; Rich and Myrold 2004) have revealed functional differences in enzyme activities and stability of microbial communities present in soil. These results were complemented with functional analysis of a number of isolates (Cavigelli and Robertson 2001) and fine-scale molecular analysis of those functionally different communities (Stres et al. 2004). Even more information could be gained if such studies could be augmented with additional fine-scale molecular diversity data of natural populations carrying out the key processes (Nogales et al. 2002). However, little methodological progress has been made in this direction, as methods are still needed to provide more in-depth and fine spatial scale resolution, e.g. eco-type resolution of microbial processes.

Numerous studies have used BIOLOG GN substrate utilisation plates to examine the diversity and evenness of substrate use of microbial communities. However, as with other culture-based methods, the resulting profile probably does not accurately represent the functional diversity of the natural microbial community (Smalla et al. 1998). For example, the response of fast-responding, heterotrophic microorganisms may predominate. An alternative technique that avoids culture biases in assessment of functional diversity is the short-term measurement of carbon dioxide respiration after the addition of selected carbon substrates to the soil environment (Degens et al. 2001; Campbell et al. 2003). This approach was further refined by

Campbell et al. (2003) who miniaturised the assay to fit available microplate reader technology. One measure of community response, CLPP (community level physiological profiling; Degens and Harris 1997; Mills and Garland 2002) has been applied as a measure of community niche width where it revealed that decreases in organic carbon reserves in soils can reduce the catabolic diversity of the soil microbial community (Degens et al. 2000). A similar technique employed an oxygen-sensitive fluorophore (Garland et al. 2003) which offers a lower potential for selective enrichment than the presently employed technology for carbon dioxide monitoring or redox dye reduction. These methods, however, do not resolve fine-scale community function that probably corresponds more directly to community structures.

There are additional variants in functional traits possessed by particular organisms isolated from the environment. Interactions, such as crossfeeding or competition among isolated strains representing dominant populations in an ecosystem and their separate growth rates, and other kinetic parameters may provide crucial information for understanding functional processes in microbial communities. Also, soil microorganisms are regularly subjected to fluctuating water potentials forcing release of intracellular solutes (amino acids, carbohydrates) into the surrounding environment thus selectively hampering strain culturability (Halverson et al. 2000). The ability to be mobile is one of the most impressive attributes of a microbe's physiology that counteracts the predator's efficiency. Cyclical dynamics of soil bacteria and their predators occur out of phase in different microniches in soil but can be synchronised and observed after strong disturbances (Zelenev et al. 2004). Mamilov et al. (2001) have shown that availability of substrates is an important factor controlling stimulation or suppression of soil microbes by soil fauna thus affecting functional properties of the soil community.

The importance of mobile genetic elements to bacterial adaptability, diversity and functional capabilities for many populations is becoming more widely acknowledged (Stokes et al. 2001; Van Elsas and Bailey 2002; Blair et al. 2004). Plasmids, phages, transposons, and gene cassettes offer venues for horizontal transfer of genes with known and unknown function. The emergence of multiple antibiotic-resistant strains in the environment is the best recognised example, and illustrates the evolution and adaptation of microbial communities to new selective forces (Krasowiak et al. 2002; Smalla and Sobecky 2002; Sengeløv et al. 2003). In this respect, the physiology of microorganisms should also be considered to a greater depth to be able to understand regulation of microbial cells under ever-changing availability of resources that drive microbial community response to environmental perturbations. To gain this level of understanding, pure cultures of targeted populations in the environment are needed. Therefore, more cultivation of yet uncultured microbes is needed. Recent advances have shown that even

simple plate count techniques with prolonged incubation times (Janssen et al. 2002; Joseph et al. 2003) or high-throughput culturing approaches (Connon and Giovannoni 2002) yield numerous representatives of microbial groups that have been represented only by sequences directly recovered from the environment or by one or a few cultivated strains. These examples clearly demonstrate that further work on cultivation and characterisation of representatives of microbial communities should be conducted incorporating recent findings on enhanced cultivation caused by additives such as cAMP or derivatives of homoserine lactones (HSL; Bruns et al. 2002, 2003). Zengler et al. (2002) have presented a new isolation approach by encapsulating cells in gel microdroplets for massively parallel microbial cultivation under low nutrient flux conditions, followed by flow cytometry to detect microdroplets which grew microcolonies. The patterns of environmental distribution and growth response of members of the uncultivated bacterial divisions offer clues for attempts to isolate and characterise division representatives in pure culture (Hugenholtz et al. 1998; Harris et al. 2004).

There is an impressive number of functional traits that can be indicators of changes in functional aspects of microbial communities. However, among all measurable traits, only a small number is used in standardised assays on a regular basis and to directly probe fine-scale community structure–function relationships. Hence, like community structure measurements, much work on functional measures at the appropriate resolution for soil lies ahead.

1.4 Newer Approaches for Linking Function with Phylogeny and Structure

The relationship between community structure (composition, diversity [richness, evenness]) and function is largely unknown. In recent years methods applying 16S rRNA-based technologies have provided crucial information on the vast diversity present in the environment. However, as is now well known, very little information on the functional role of detected taxa can be retrieved from the use of 16S rRNA genes as ribosome content is only weakly correlated to metabolic activities (Klappenbach et al. 2000; Forney et al. 2004).

As many new gene sequences are retrieved directly from the environment by different molecular techniques, functional analysis without cultivation has become possible. While the function of some cloned genes can be inferred by sequence comparisons, measurement of the activity of the encoded protein is needed for proof of function. Substrate specificity is difficult to infer from sequence and a number of proteins have more than

one function, both challenges for interpreting DNA sequence information. Despite difficulties expressing environmental genes in heterologous hosts there have been many studies that utilise expression to confer protein function (Rondon et al. 2000; Gillespie et al. 2002; Martinez et al. 2004). This also allows identification of genes of biotechnological or medical importance and occasionally helps with determining the function of those genes with previously unknown function.

The approach exploiting the use of bacterial artificial chromosomes (BAC; Rondon et al. 2000; Liles et al. 2003; Martinez et al. 2004) considers all genomes of microorganisms in soil to present a metagenome – one large soil microbial community genome. Comparison of 16S rRNA genes with upstream and downstream open reading frames (ORFs) located on large BAC inserts has revealed a huge diversity on fragments harbouring almost identical 16S rRNAs. This provides information not only on phylogenetic but also functional diversity. Comparison of closely related Archaea originating from a single population revealed significant genomic divergence that was not evident from 16S rRNA sequence variation. This approach has directly revealed the existence of considerable functional diversity within single populations of coexisting microbial populations, even those with identical 16S rRNA gene sequences. The use of arrays to screen large BAC insets, i.e. metagenomic profiling – for genes of known function (Sebat et al. 2003) – further extends the usefulness of this approach.

As an alternative, reconstruction of genomes directly from environmental DNA is a technically and computationally demanding approach. However, the tedious and often cumbersome process of isolation and cultivation of microorganisms in pure culture is obviated by the use of conventional random shotgun sequencing of environmental DNA. After isolation of microbial biofilm DNA, 76.2 Mbp were sequenced; the genomes were assembled at coverage up to 10× that allowed for high-quality assembly and resulted in two complete genomes and three with slightly lower coverage, thus enabling an insight into the metabolic pathways of all five organisms representing the majority of the community (Tyson et al. 2004). The effectiveness of this approach in other environments, especially soil, is definitely limited by high species richness, heterogeneities in abundance of community members, as well as by extensive genome rearrangements. However, even in complex environments, it should be possible to extend random shotgun sequencing to recover large segments of genomes of uncultivated species, as was recently the case (Venter et al. 2004). Without some selective recovery approach, it is likely that low-abundance species, which are nevertheless involved in important processes, will be overlooked. The ability to retrieve full genome sequences from the environment presents a platform for inferring microbial genetic networks within a single genome sequence (Weng et al. 1999; Gardner et al. 2003). This approach can also provide

crucial information to find conditions for cultivating previously uncultivated organisms, to construct DNA microarrays to monitor community structure over time, or to monitor community gene expression. Further, the full genome sequences of dominant community members can be used to explore the nature of the community metabolic network (Weng et al. 1999; Gardner et al. 2003; Karaoz et al. 2004). This is an integrative bioinformatic approach of compiling data from microbial genomic networks to understand interactions at community levels (Vallino 2003). However, a large fraction of newly discovered genes have an unknown functional role and this is thought to be a major barrier to comprehensively understanding a cell's metabolic machinery. Nonetheless, integration of functional information is a promising direction for improving the accuracy and robustness of functional genomics (Karaoz et al. 2004) that can be further exploited in construction of microarrays. The comparative and quantitative analysis of expressed mRNAs of key enzymes in relation to 16S rRNA is beginning to provide information on functional expression of genes of novel microbes in relation to environmental factors. Attempts were also made in such directions by applying array technology to linking specific metabolic pathways to their ecological function (Nogales et al. 2002). Nonetheless, it seems that ascribing a microbial process to a specific microorganism remains an elusive project, as many microbes operating distinct processes do so in cooperation. Before organisms are isolated in pure cultures, their ecological functions need to be characterised through (classical) culture-independent characterisation, linking phylogeny and functional genetic analyses to metabolic activities in soil using the approaches mentioned above and also including those described below.

Microradioautography and fluorescent in situ hybridisation (MICA+FISH) make use of short-term uptake of specific radiolabelled substrates by individual cells to identify individual cells, to determine its three-dimensional position by FISH, and to quantify active populations utilising the substrate of choice by microradioautography (Lee et al. 1999; Adamczyk et al. 2003; Polz et al. 2003). The microradioautography approach was recently further refined by the use of microarrays for hybridisations of DNA derived from incubations of microbial communities with ^{14}C-labelled substrates. After DNA hybridisation digital image analyses of array blots and autoradiographs are performed to extract quantitative information (Adamczyk et al. 2003). Arrays, however, suffer from quantitative difficulties, and, if such analyses are performed only on the 16S rRNA gene, the mere incorporation of ^{14}C into rRNA corresponds only weakly to synthesis of new ribosomes.

As an alternative, stable isotope probing (SIP) relies on labelling the DNA of an active (growing) microbial community enriched on ^{13}C-labelled substrate. ^{13}C-enriched DNA can be resolved from ^{12}C-DNA by density

gradient centrifugation and each DNA fraction can be further analysed taxonomically and functionally by classical gene analysis. In addition to labelled DNA, other cell constituents can be used as biomarkers, such as ^{13}C-PLFA, but the resolution for diversity analyses is less powerful than by sequence analysis (Bull et al. 2000; Radajevski et al. 2000).

Another non-isotopic method that can be used to link activity to phylogenetic information is the incubation of environmental samples in the presence of bromodeoxy uridine (BrdU) which is incorporated by active community members. The BrdU in the DNA can be later immunocaptured and separated from the unlabelled DNA using magnetic separation. The extracted DNA fractions are subjected to either profiling or cloning and sequencing, and the number and diversity of bacterial groups responding to stimuli can be determined (Borneman 1999; Yin et al. 2000).

The common drawback of MICA+FISH, SIP and BrdU is that a sample is incubated with labelled substrates and therefore prone to selective activation of cells with substrates under different conditions than were present in situ. Microbes are able to slightly discriminate between different isotopes of the same element (Londry and Des Marais 2003) and some do not take up BrdU. However, all these methods offer a powerful advantage of at least distinguishing the active from the extensive inactive background community in soil.

Although uncommon, the examination of community functional characteristics across a series of dilutions and re-growth stages offers a promising way of generating communities differing in diversity (richness and evenness) and varying in overall community structure. As numerical algorithms for simulations were also developed (Franklin et al. 2001; Griffiths et al. 2004), the use of this method in conjunction with other molecular and classical techniques may be a useful approach for the study of microbial diversity and related ecological parameters such as niche width and metabolic redundancy (Garland and Lehman 1999; Franklin et al. 2001; Griffiths et al. 2004).

While there are many methods that enable linking of microbial phylogeny with function in soil environments, there is still a huge need for methodological improvement.

1.5 Future Challenges

The approaches briefly mentioned here are at least partially flawed and at best provide an incomplete and perhaps distorted view of microbial communities. In this respect, the major weakness of metagenomic and direct genome reconstruction and also current PCR approaches is that they may

overlook parts of the community that are in low abundance but nevertheless perform crucial processes. An enrichment strategy is needed to focus on genomes coding these defined processes. There is a potential to use SIP in combination with mRNA, metagenomic or even direct genome reconstruction approaches to relate the functional capabilities of the microbial community to the biodiversity present within it. Most of the approaches mentioned above are also dependent on quantity and quality of sequence database data that serve as a starting point for primer and probe design and also for affiliation of detected sequences. The number of sequences has been increasing dramatically; however, the main sequence databases are not curated in a systematic way. Therefore, the balance between universatility and specificity of 'universal' and degenerate primers and PCR conditions should be constantly improved to identify the combination that results in the most meaningful and comprehensive apparent diversity. Whole groups might be excluded depending on the primers chosen although they can be relatively easily improved as sequence data abounds also for functional genes. It is also time to shift focus from the 16S rRNA gene as the dominant measure of diversity toward other genes as the 16S rRNA gene can be no better than a secondary measure when functional understanding is the goal. Isolation of novel strains from the environment and characterisation of genes of choice may prove an excellent means to improve molecular primers and probes to allow for lower and much broader detection limits of target sequences in environmental samples. In this respect, fine-scale diversity analyses of functional genes are needed. While there is no apparent and simple way to determine if extracted DNA or RNA is representative (as yield per se is not sufficient), one should keep in mind that a significant bias may be introduced during the isolation of genomic DNA and RNA and interpret the results accordingly.

In this respect, the differences observed in microbial community DNA fingerprints are most commonly explained by a difference in species richness due to an increase in a number of DNA fragments detected. This would mean that one community is more diverse and distant from the other community. However, such differences could be completely due to differences in rank-abundance of populations with no real change in species richness. The loss of a fragment from a fingerprint can therefore be due to a change in a detectability of a target sequence. Further, the snapshot of diversity where microbial diversity is assessed at only one time in one location is not terribly informative (Kent and Triplett 2002). Where diversity over time, space and gradients of soil chemistry has been measured, the dynamic nature of microbial communities can be observed and the presence or absence of individual populations can be compared with changes in ecosystem processes. However, seeing patterns of diversity changing (DGGE, TGGE or T-RFLP) with various treatments is not sufficient if identification and

isolation of organisms with fluctuating presence or absence dynamics is not attempted and their functional role in microbial processes elucidated in response to ecologically relevant factors. Therefore, more effort should be directed toward the isolation of representatives from newly detected and ubiquitous bacterial groups. Recent cultivation successes suggest that if more effort were put into isolation of bacteria in pure cultures, more of them would prove culturable, especially as little research is conducted on unspecialised, aerobic and heterotrophic bacteria. Another common pitfall is that many studies retrieve sequences from the environment, compare them with databases and conclude that the lack of match in the database indicates that the organism has not yet been cultured. Such conclusions ignore the fact that less than half of the type cultures have yet to have their 16S rRNA determined (Hugenholtz et al. 1998) and that many thousands of poorly characterised and misidentified strains are maintained in culture collections throughout the world. In addition, the lack of consensus regarding the prokaryotic classification on phylogenetic data at the species level introduces a degree of uncertainty into published results. Stackebrandt and Goebel (1994) concluded that if 16S rRNA sequences have less than 97% identity then the sequences are most likely to be derived from different species. Further, if the sequences have more than 97% identity, then they may either come from the same species or from different species, and in this case DNA–DNA hybridisation analyses are required to establish the species relationship. It is not at all uncommon for the 16S rRNA sequences of distinct bacterial species to be >97% similar. There is published evidence where their findings were misinterpreted asserting that >97% identity of two 16S rRNA sequences is sufficient to conclude the two strains derived from the same species. The '97% rule' misinterpreted in such a manner is incorrect, inappropriate and inaccurate for many prokaryotic species and much care should be taken to avoid such misleading conclusions in the future (Stackebrandt and Goebel 1994; Forney et al. 2004).

More than 200 microbial genomes are already sequenced and hundreds more are in the process of being assembled. This means a huge amount of sequence information is arriving about novel metabolic pathways and drug resistances, gene regulatory elements and networks, pathogenicity, virulence and other ecological niche specialisation systems, and also of genes with unknown function. Species diversity based on comparative genomics has opened a new window into evolution of genes, species and plasticity of genomes. Future research should be directed toward extracting valuable information from a huge amount of evolutionary and functional data and to ask questions that are important in microbial ecology. In this respect, DNA and mRNA microarray technology should concentrate on differential expression of genes to gain clues about function of genes with unknown function, to assess species diversity and the relationships between active

populations with target genes in relation to environmental parameters, and to provide guidelines for designing new and improved culturing and molecular methods (DeLong 2002).

Long-term ecological research (e.g. LTER) sites provide an excellent means for relating function–structure of microbial communities as these sites have long-term data on the temporal and spatial variability of a wide range of physical, chemical, and biological properties. In this respect, long-term data allow the researcher to correlate changes in microbial communities with ecosystem processes (Cavigelli and Robertson 2000; Robertson et al. 2000; Buckley and Schmidt 2001, 2003; Bergsma et al. 2002; Stres et al. 2004). It is also important to devise statistically valid sampling and to have a sufficient number of samples taken within each treatment group so that hypotheses can be statistically validated, as this is almost absent from studies of microbial ecology.

In this respect it is also time to put more emphasis on rigorous modeling of processes in vitro on laboratory scales or in controlled field experiments to identify the effects of factors governing processes in the field, especially as modeling offers a higher degree of experimental control. The ultimate test of our ecological understanding is to predict the behaviour of an ecological system. The inability to predict accurately the behaviour of simplified laboratory systems signifies the lack of our understanding to make predictions on the field scale. Because of their rapid generation times, microbial model systems can be used to address ecological questions over multiple temporal scales and also allow exploration of community-level responses to environmental change (Jessup et al. 2004; Palumbo et al. 2004).

There are conflicting theories on the importance of species diversity in relation to functional resistance and resilience of soil microbial communities. Dilution studies (Garland and Lehman 1999; Griffiths et al. 2004; Mei et al. 2004) have shown that linear changes in microbial diversity may result in non-linear changes in ecosystem functioning. Other studies have shown that diversity peaks at intermediate ecosystem productivity (Kassen et al. 2000) and that resilience assessments depend strongly on the parameter investigated (Pesaro et al. 2004). Genomic diversity and ongoing diversification and specialisation allow for functional redundancy and parallel substrate metabolism to develop and provide grounds for functional stability. During the past two decades, the advancement of soil molecular ecology has resulted in an uncoupling of microbial studies from measurements of ecological processes, soil physics and chemistry, as numerous reports describe only snapshots of diversity without accompanying relevant ecological data. While this may be a reasonable first phase to establish depth and rigour to the new microbial measures, it is time to couple these approaches to process and habitat measures to begin to elucidate the structure–function relationships. An integrated approach to studying

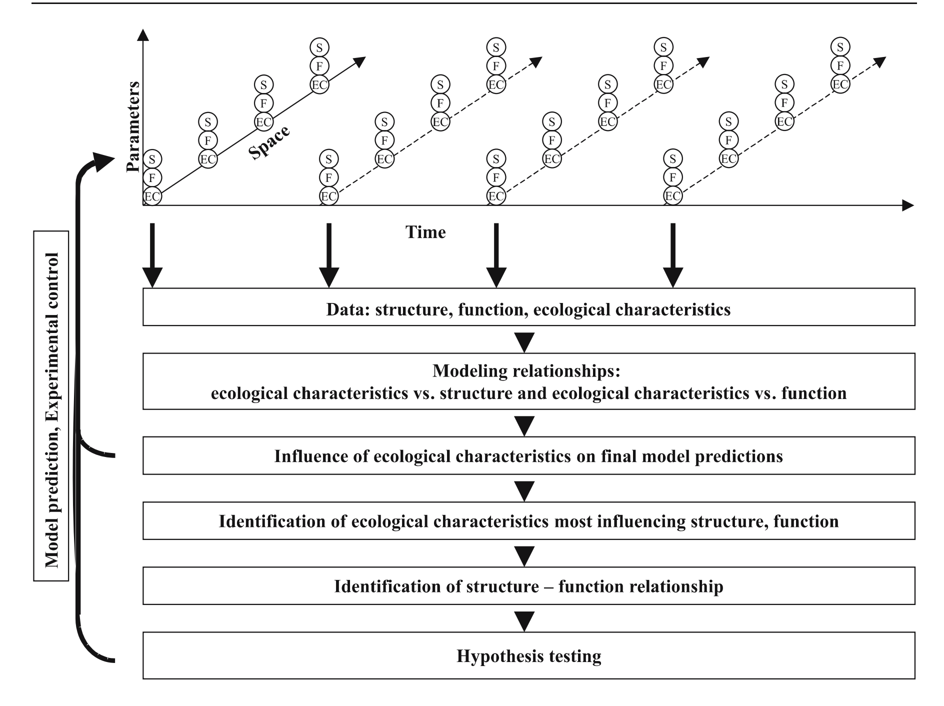


Fig. 1.1. Coupling of structural (S), functional (F) and ecological characteristics (EC) data to identify underlying relationships. Multiple sampling points in space and time provide a data set encompassing community structure, functional aspects and environmental parameters all serving as input to the selected model to elucidate EC:S and EC:F relationships and is validated to identify the ECs that have the greatest influence on the predicted S and F values. Through the comparison of EC:S and EC:F relationships the S:F relationship can be deduced allowing hypotheses to be formed that can be further tested by comparing the model predictions with additional experimental data sets

soil with emphasis on the interactions between physical and biological processes is required for incorporating functionality, ecosystem dynamics and evolution at its core. Various methods of analyses, multiple sampling points in time and space, and also modeling of ecological and global factors are envisioned (Fig. 1.1). In this respect, to identify the critical factors that influence population distribution and activity in complex environments more sophisticated statistical and mathematical techniques are needed to model the relationships between microbial community structure, function and environmental characteristics. However, the complexity of interactions and scale are the major obstacles on our path towards understanding the relationships between structural and functional aspects of microbial communities and environmental characteristics. Determining what environmental characteristics exert strong statistical influence or coincide with specific populations (structure and function) may be hampered by the fact

that the critical environmental component may not always be measured or at least not on the appropriate scale (Palumbo et al. 2004).

Finally, there is a compelling published literature on biodiversity showing that, in discussions of biodiversity, the use of the same ecological language is needed. Macroecologists define biodiversity in terms of species and differentiate diversity on three scales: α (within site), β (between sites), and γ (across a landscape). The diversity within a given site is defined according to three long-known parameters: species richness as the number of species in an area, species evenness or equitability as the relative abundance of species, and, lastly, species composition as the description of actual species [or operational taxonomic units (OTUs) in molecular ecology] that are present in a sample (Magurran 1988; Forney et al. 2004). In this respect, the standardisation of the methodology used for environmental studies and rigorous use of statistical evaluation of results obtained from the environmental studies are advocated to establish grounds for hypothesis testing and intercomparisons of results that the current microbial ecology lacks almost entirely.

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2 Chemical Structure of Organic N and Organic P in Soil

Ingrid Kögel-Knabner

2.1 Introduction

Nitrogen (N) is a key element in controlling the functioning, dynamics and diversity of terrestrial ecosystems. Organic N in terrestrial systems occurs primarily in dead organic matter and humic substances of soils (Schlesinger 1991; Stevenson 1994), comprising about 96% of the total. The remaining 4% of the total terrestrial organic N occurs in living biomass (Stankiewicz and van Bergen 1998). The terrestrial living biomass organic N is dominated by plant N with 94–99%; only 1–6% is part of animals and microorganisms (Stevenson 1994).

Organic N incorporated into soil organic matter (SOM) represents a major reservoir of N on the earth's surface. Its primary source is biochemical N from plant and animal residues (predominantly proteinaceous substances), which undergo a complex series of transformations, mediated by microbial and abiotic processes, ultimately resulting in the stabilisation of the non-mineralised N fraction in soils (Schlesinger 1991; Stevenson 1994; Schulten and Schnitzer 1998). During these stabilisation processes the biochemical N is thought to be extensively altered structurally, forming more stable compounds, such as humic substances or other stabilised geomacromolecules, although the type of changes in chemical speciation, their timing, and mechanisms are not clear (Knicker 2004). The concentration of total N in top soils varies widely, depending on the organic carbon (OC) content, ranging from 1 to 2 $g\,kg^{-1}$ in agricultural top soils. Higher concentrations are reported for grassland and forest soils. More than 95% of the total N in soils occurs in the form of organic N compounds (Bremner 1965). Most of the inorganic forms of N are susceptible to immediate loss from the soil in the form of dissolved NO_3^- or gaseous N_2O or NH_3. Only NH_4^+ is reversibly adsorbed to clay minerals or fixed between mineral lattices of vermiculite, illite and montmorillonite (Stevenson 1994). Thus, especially in the subsoil, substantial proportions of N may occur in the form of NH_4^+ adsorbed or fixed in clay minerals.

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Nucleic Acids and Proteins in Soil
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When the turnover time of N in the soil is calculated with respect to the input of dead plant materials, the mean residence time of N in soils is about 50 years (Schlesinger 1991). In comparison, for the total pool of OC in soils a mean residence time of about 26 years (Schlesinger 1991) or 40 years (Oades 1988) was estimated. This indicates that N, mostly in the form of organic N, is conserved in soils. Evaluating these numbers one has to bear in mind that the mean residence time of organic material in soil varies over several orders of magnitude between the surface litter and the various humus fractions.

Organic phosphorus (P) is also a major component of total soil P (Magid et al. 1996). Nonetheless, it remains poorly characterised and the role of organic P forms in plant nutrition and the P cycle is not well understood. Concentrations of total P in top soils range between 100 and 900 $mg\,kg^{-1}$ (Stevenson 1994). The continued application of P fertilisers and manures in amounts exceeding plant requirements leads to an accumulation of P in top soils under agriculture. Organically bound P constitutes often more than 50% of the total P, although it may range from as low as 15–20 to more than 80–90% (Tate 1985; Stevenson and Cole 1999). The mean residence time of organic P in soils is estimated between 350 and 2000 years (Paul and Clark 1996).

Today, our understanding of the chemistry of organic N and P compounds in soils is far from complete. It is important to have this knowledge because the type of N and P formed in soils influences not only its reactivity and fate (e.g. the release of bioavailable N in soils), but also because many of these compounds have important functions in the biochemical regulation of soil processes. Nucleic-acid-based and chemical-biomarker-based methods are now widely used for assessing soil microbial communities, without the need for isolation and cultivation. This chapter will provide an overview of the chemical structures of organic N- and P-containing compounds derived from plant or microbial remains in soils and the methods used to characterise them.

2.2 Biological Forms of Organic N and P That Enter Soils

In soils the major part of the organic N derives from decaying vascular plant material. Additional sources of N-containing compounds are the residues from the microbial biomass. Detailed descriptions and accounts of the composition and structure of plant and microbial components are given by de Leeuw and Largeau (1993), Stevenson (1994), Paul and Clark (1996), and Kögel-Knabner (2002). Of the plant dry mass, 2–15% is assigned to N-containing compounds (Haider 1992). Nitrogen is a compo-

nent of three very important biological macromolecular structures, i.e. proteins/polypeptides, polymers of amino sugars and DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). Minor biological sources of organic N are smaller molecules, such as porphyrins (mainly chlorophyll and hemoglobin) and various N-containing secondary plant metabolites, such as alkaloids and sphingolipids (Stevenson 1994). Of the bacterial biomass, 50–60% can be assigned to N-containing compounds (Haider 1992). The ratio of protein to RNA is around 5 and the ratio of protein to DNA about 2. The relatively high content of N-containing biomolecules is responsible for the low C/N ratio of 5–8 of bacterial biomass (Paul and Clark 1996). Fungi contain approximately 14–52% N-containing compounds. Most of the N-containing compounds in plants and microorganisms are known to be highly sensitive to microbial degradation and are therefore expected to be quickly mineralised and/or reutilised for biological production.

Rhizodeposition, i.e. all organic matter released by living roots, accounts for a substantial input of organic N in soils (Whipps 1990). The amount of data for soils is limited, mainly because most of the data obtained from sterile soil experiments or nutrient solutions cannot be applied to natural soils. Root exudates containing N are mainly amino acids and amides, but nucleotides and flavonones are also reported (Uren 2001; Hütsch et al. 2002; Kuzyakov et al. 2003). Most of the exudates are rapidly consumed by soil microorganisms and subsequently immobilised in microbial residues (Mayer et al. 2003).

The majority of organic P inputs into soils from both plant and microbial origin are in the form of orthophosphate diesters (Turner et al. 2002). Microbial inputs are dominated by nucleic acids with 60% of total P and phospholipids, accounting for 5–30% of the microbial and fungal input, and even more in plant input to soils.

2.2.1 Proteins and Polypeptides

In living organisms most of the organic N is in the form of protein-bound amino acids. Proteins serve manifold purposes, e.g. as enzymes, transport proteins, regulators, storage substances or as structural proteins, and are usually composed of the 20 most frequent amino acids, which can be subdivided into basic, neutral or acidic amino acids (Fig. 2.1). In addition to the 20 major amino acids of proteins, bacterial cell walls also contain a series of unusual amino acids, linked in a two-dimensional structure which provides rigidity and elasticity to the bacterial cell wall (Koch 1990). The proteins from plant and microbial tissues can be decomposed by a multi-

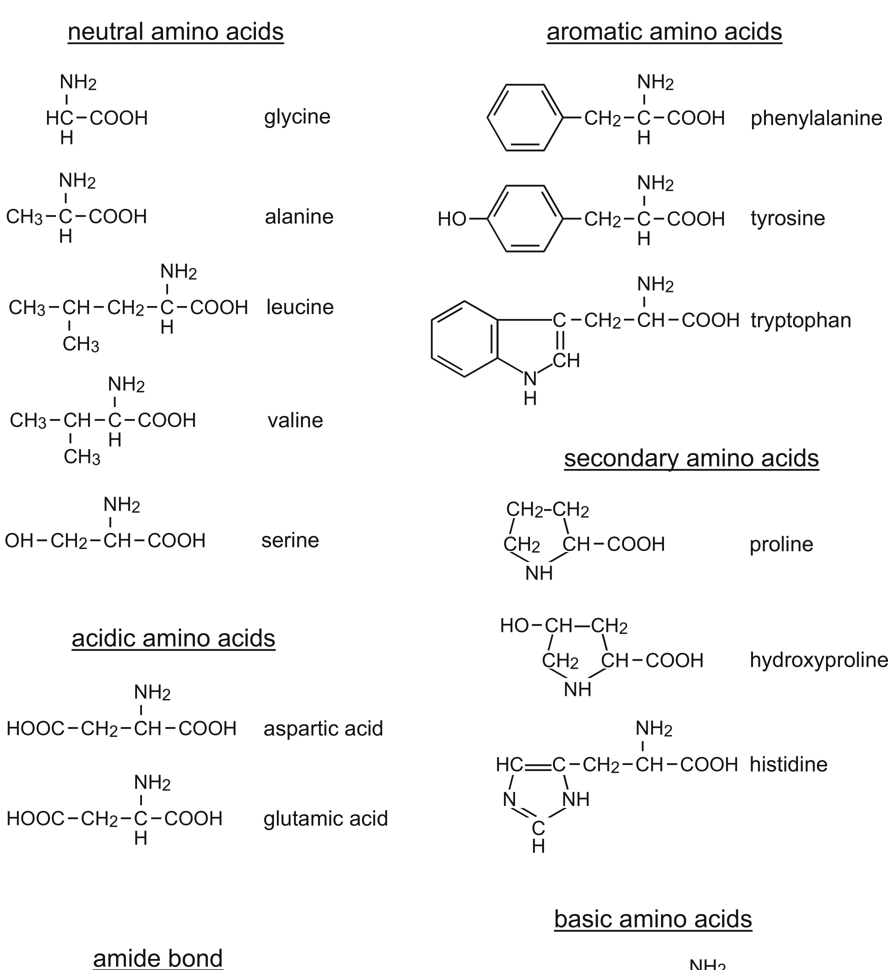


Fig. 2.1. Examples for the chemical structures of neutral, acidic, basic, aromatic and secondary amino acids and the amide bond

tude of microorganisms and are considered to be less stable components with high turnover rates, due to the easily decomposable amide linkage (de Leeuw and Largeau 1993; Derenne and Largeau 2001). Nonetheless, there is growing evidence that enzymes and other protein-type materials may survive over extended time spans in soils (Nannipieri et al. 1990; Ladd et al. 1996; Amelung 2003; Knicker 2004).

2.2.2
Amino Sugar Polymers

The basic unit of the cell walls of fungi and also of the exoskeleton of insects is chitin (Peberdy 1990). It is a highly ordered polysaccharide of (1–4)-linked 2-acetamido-2-deoxy-*β*-D-glucose (N-acetylglucosamine; Fig. 2.2). Chitosan is composed of the deacetylated form (2-amino-2-deoxy-*β*-D-glucose).

Eubacterial cell walls are composed of a peptidoglycan (also called murein or mucopeptide), which contains carbohydrate as well as amino sugar and amino acid elements (Fig. 2.3). Whereas glucosamine is also found in insects and fungi, galactosamine, muramic acid and diaminopimelic acid are only found in bacteria (Stevenson 1994).

Mureins are peptidoglycans consisting of polysaccharide chains with short peptides attached (Fig. 2.4). The carbohydrate backbone of murein

chitin

Fig. 2.2. Structure of chitin

β-N-acetyl-D-glucosamine

β-N-acetyl-D-galactosamine

N-acetyl muramic acid

meso-diaminopimelic acid

Fig. 2.3. Structures of major amino sugar compounds (glucosamine, galactosamine, muramic acid) and diaminopimelic acid found in soils

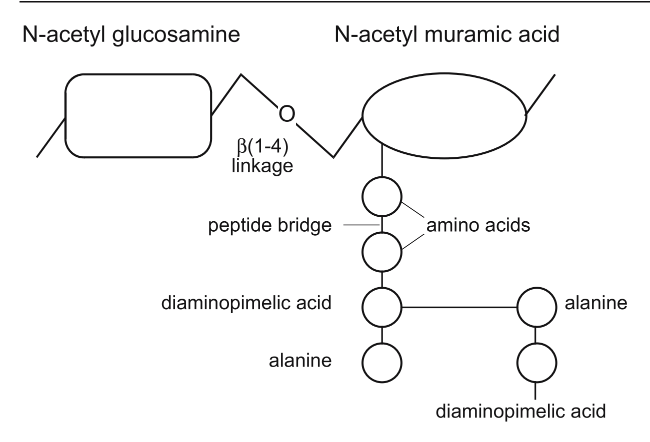


Fig. 2.4. Structure of a peptidoglycan (murein) subunit

is composed of alternating units of 2-acetamido-2-deoxy-*β*-D-glucose (*N*-acetylglucosamine) and 2-acetamido-3-*O*-(1-carboxyethyl)-2-deoxy-*β*-D-glucose (*N*-acetylmuramic acid). The cross-linking of the chains occurs via peptide bridges (de Leeuw and Largeau 1993). The composition of the peptides varies considerably between Gram-positive and Gram-negative bacteria, and also between individual species. They contain common amino acids, but also specific amino acids, such as D-glutamic acid and D-alanine, diaminopimelic acid and D-lysine (Koch 1990). Cell walls of Gram-positive bacteria contain approximately 20–40 murein layers, whereas the cell walls of Gram-negative bacteria are composed of fewer, even possibly only one murein layer. Therefore, murein amounts to approximately 50% of the dry weight of the Gram-positive cell wall, but only 10% of the dry weight of the cell wall of Gram-negative bacteria (Koch 1990).

2.2.3 DNA and RNA

Organisms use nucleic acids for the storage of genetic information (DNA) and for the translation of this genetic information for protein synthesis (RNA). The nucleotides forming DNA and RNA are composed of a phosphate, sugar and heterocyclic N-containing base unit, i.e. the purine units adenine, guanine and the pyrimidine units uracil, cytosine, and thymine (Fig. 2.5). Outside living cells, RNA and DNA are considered to be biodegraded very easily (de Leeuw and Largeau 1993). In soils they are susceptible to adsorption onto clay minerals as well as humic materials. These surface interactions allow them to persist in the soil environment, but their bioactivity may be diminished (Chenu and Stotzky 2002). For further details, see Chaps. 6 and 7.

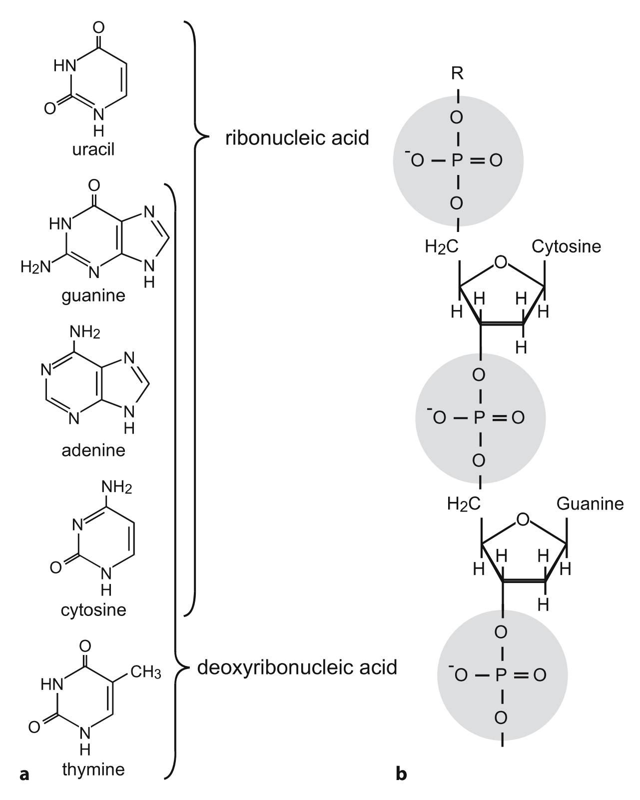


Fig. 2.5. Basic structural units of DNA and RNA: (**a**) purine and pyrimidine bases and (**b**) sugar-phosphate backbone

2.2.4 Minor N-Containing Molecules

Chlorophyll consists of four pyrrole rings which, together with a fifth ring, build a porphyrin structure (Fig. 2.6). In most chlorophylls a long phytol chain is bound to the porphyrin rings (Hendry 1988). Chlorophyll is present in all photosynthetically active cells in higher plants, algae and bacteria. The different forms of chlorophyll are obtained from modifications in the side-chain substitutions at the ring structure.

2.2.5 Teichoic Acids

Teichoic acids are acidic mucopolysaccharides in the cell wall of Gram-positive bacteria with a phosphodiester structure (Fig. 2.7). They consist

Fig. 2.6. Basic porphyrin structure of chlorophyll built from pyrrole rings. The phytol side chain is linked via an ester bond

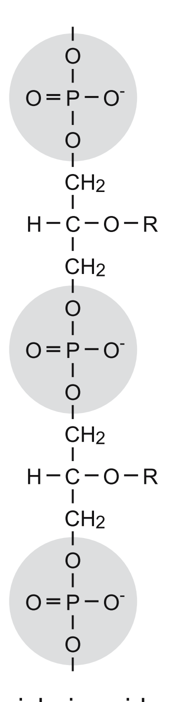


Fig. 2.7. Structure of organic P components: teichoic acids

of repeating units of glycerol or ribitol and are connected by phosphate esters (Shockman and Barrett 1983; de Leeuw and Largeau 1993). Teichoic acids are covalently attached to the murein in the cell wall via the phosphodiester linkage. The teichoic acids can be partly replaced by teichuronic acids, polymers consisting mainly of D-glucuronic acid and *N*-acetylgalactosamine.

2.2.6
Inositol Phosphates

Inositol phosphates are synthesised mainly by plants, but small amounts are also reported to be found in bacteria and fungi (Turner et al. 2002). Inositol phosphates are esters of hexahydroxycyclohexane (Fig. 2.8). In plants inositol hexaphosphates are mainly found in storage organs in the form of Ca or Mg salts, called phytin. Depending on the arrangements of H and OH groups, nine positional isomers are possible (Stevenson and Cole 1999). In addition to inositol hexaphosphate, other inositols only partly esterified with phosphates are found in plants and soil.

monophosphoinositol

Fig. 2.8. Structure of organic P components: monoinositol phosphate; all OH groups can carry a phosphate group, giving phytate, the hexaphosphate ester of inositol

2.2.7
Phospholipids

The membranes of all living organisms contain a phospholipid bilayer. The phospholipids are mostly derivatives of glycerin with phosphodiester structures, sometimes also phosphomonoesters (Fig. 2.9). In the phosphodiester structures the phosphate group is bound to amino alcohols, such as cholin (lecithin), 2-aminoethanol, or L-serine. These components are released during hydrolysis of phospholipid extracts from soils. Phospholipids in soils are mainly of microbial origin and are predominantly phosphatidyl cholines, followed by phosphatidyl ethanolamines (Stevenson and Cole 1999). In archea, which also play a role in soils, ether-linked lipids are found. The fatty acid pattern of phospholipids is specific for different bacteria or fungi and is used for community characterisation (Table 2.1). This implies that such compounds should only be released from living cell materials and are not stable in soil (Zelles 1999; Insam 2001). The outer membrane of Gram-negative bacteria contains a specific lipopolysaccharide composed of a core polysaccharide and a lipid substructure (the so-called lipid A), that contains phosphate groups attached to glucosamine in a monoester-type bond.

$CH_2OC(=O)R_1$ – $CHOC(=O)R_2$ – $CH_2OP(=O)(O^-)–OH_2CH_2\overset{+}{N}(CH_3)_3$

lecithin

$CH_2OC(=O)R_1$ – R_2COOCH – $H_2C–O–P(=O)(O^-)–CH_2CH(COO^-)\overset{+}{N}H_3$

phosphatidyl serine

$CH_2OC(=O)R_1$ – R_2COOCH – $H_2C–O–P(=O)(O^-)–OCH_2CH_2\overset{+}{N}H_3$

phosphatidyl ethanolamine

Fig. 2.9. Structure of major phospholipids: R1 and R2 are different fatty acids, as given in Table 2.1

Table 2.1. Specific phospholipid fatty acid components of the soil microbiota (from Insam 2001)

Microbial group	Phospholipid fatty acid signatures
Archaebacteria	Ether-linked fatty acids
Anaerobic bacteria	Sphingolipids, largely absent from aerobes
Bacteria in general	Ester-linked monounsaturated fatty acids
Gram-negative bacteria	Contain more hydroxylated fatty acids
Gram-positive bacteria	Contain more branched fatty acids
Cyanobacteria and eucaryotes	Polyunsaturated fatty acids
Fungi	Specific fatty acid (18:2ω6)

2.3 Techniques To Analyse Soil Organic Nitrogen

A large proportion of the soil organic N (SON) has not been identified yet on a molecular basis. This is mainly due to the problems of isolating these compounds from soil by chemolytic, thermolytic or combined thermochemolytic methods (Stevenson 1994; Leinweber and Schulten 2000). Alternative techniques for the examination of organic matter in heterogeneous macromolecular mixtures are non-destructive spectroscopic methods, which include nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy and X-ray absorption near-edge structure (micro)spectroscopy (XANES, also known as NEXAFS; Kögel-Knabner 2000; Sparks 2001). The big advantage of such techniques lies in the fact that the sample can be analysed without major pretreatment and extraction. The sample can be examined as a whole. Most of these methods, however, are relatively insensitive and reveal low resolution. Although these techniques can give good results concerning the gross chemical composition, specific compounds are hardly identified.

2.3.1 Hydrolysis

Acid hydrolysis (usually with 6 M HCl), used to release amino acids from proteins, is also often used to liberate N compounds from soil (Bremner 1949, 1952). Bremner (1965) and Kelly and Stevenson (1996) give a detailed description of the procedures and difficulties associated with this method. The hydrolysis is followed either by a chromatographic separation of the individual amino acids released [usually by high-performance liquid chromatography (HPLC), or gas chromatography) or by photometric determination of the total concentration of α-amino groups in the hydrolysate (Stevenson and Cheng 1970; Kögel-Knabner 1995). This method leaves about 20–35% of the total SON unattacked (Sowden et al. 1977; Leinweber and Schulten 2000). Due to its insolubility, this so-called non-hydrolysable residue is excluded from the investigation with common chemolytic approaches.

With common analytical methods between about 30–50% (Stevenson and Cole 1999) of the total SON was identified, mostly as amino acids and amino sugars. Besides amino acids and amino sugars, the hydrolysate contains nucleic acids and other N biomolecules. For their separation and identification specified techniques are required (Kelly and Stevenson 1996).

Ammonia, produced during hydrolysis, can be recovered by steam distillation with MgO and amounts to 20–25% of the N in surface soils (Kelly and Stevenson 1996). Because in most cases an unbiased assignment to the originating compounds is not possible, this fraction is commonly termed the hydrolysable unknown-N (HUN) fraction (Stevenson 1994). It partly derives from soil NH_4^+ and NH_3 liberated after degradation of amino acid amides, such as asparagine and glutamine. Another part of this N may originate from partial destruction of amino sugars, but also amino acids such as serine, threonine or tryptophan. Threonine and serine are slowly degraded to NH_4^+ and carbonyl. Tryptophan is stable if presence of air is avoided. However, it was shown that in the presence of iron(III) and copper(II) even degassing prior to hydrolysis gives no protection. An accounting of all known potential sources of NH_3 in soil hydrolysates shows that about one-half of the NH_3-N, equivalent to 10–12% of the total organic N, is still obscure (Kelly and Stevenson 1996). Some of this unknown hydrolysable N could originate from pseudoamines, such as iminoquinones, Schiff bases and enamines, hydroxyamino acids, amino alcohols and sugars. Purines and pyrimidines were also suggested as possible origins of this unidentified hydrolysed N.

2.3.2 Analytical Pyrolysis and Thermochemolysis

Analytical pyrolysis is used to study the structure of the organic N that cannot be released by hydrolysis. The thermolytic degradation releases small fragments from the macromolecular structures. Pyrolysis in combination with gas chromatography and mass spectrometry (Py-GC-MS) involves chromatographic separation of the fragments into single components and mass spectral data are obtained for each component. Only volatile compounds, the polarity of which allows chromatographic separation, can be identified. Pyrolysis-field ionisation mass spectrometry (Py-FIMS) does not involve separation of the pyrolysis products, but uses soft ionisation to produce predominantly molecular ions of the pyrolysis products (Schulten et al. 1997). The fragments are considered representative of the original larger macromolecules. The interpretation of pyrolysis data requires a detailed knowledge of the pyrolysis behaviour of the compounds under study, as many pyrolysis products can originate from chemically diverse SOM components. In addition, thermally induced secondary reactions can cause considerable modification of the original compound, which may bias the pyrolysis data. Schulten and Schnitzer (1998) give a summary on the N-containing artifacts found in soil pyrolysates, mostly heteroaromatic N compounds. They include pyrroles, imidazole, pyrazoles, pyridines, pyrazines, and indoles. These examples illustrate the complexity of pyrolysates obtained from SOM, and the difficulties involved in the interpretation of data obtained from pyrolytic studies of SOM. In most cases, a quantification is not possible.

Thermochemolysis is based on simultaneous fragmentation and methylation of the produced monomers with gaseous tetramethylammonium hydroxide (TMAH). The polar products are converted into less polar derivatives which are then more volatile and thus susceptible to chromatographic separation (Challinor 1995). With the thermochemolysis method many more structurally significant products are separated and detected than was previously possible with the conventional Py-GC-MS methods (Hatcher and Clifford 1994; Saiz-Jimenez 1994). Figure 2.10 shows an example for protein-derived thermochemolysis products. By using TMAH thermochemolysis, Knicker et al. (2001) could not identify N-containing aromatic compounds in major proportions. Schulten et al. (1997) observed N derivatives of benzene and long-chain nitriles, not usually detected in Py-MS of plants and microorganisms after pyrolysis/methylation. They suggested that these compounds result from stable transformation products of soil N and consider them as characteristic degradation products of SON structures.

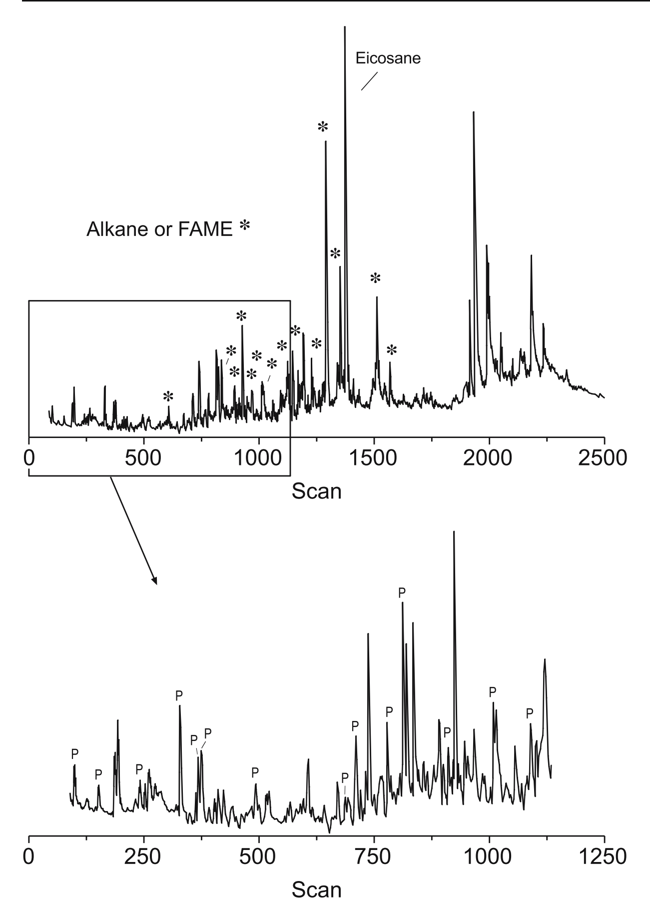


Fig. 2.10. Results obtained from the thermochemolytic treatments of SOM with TMAH; * peaks assigned to alkanes and fatty acid methyl esters, P peaks assigned to protein-derived compounds (from Knicker et al. 2001)

2.3.3 Solid-State ^{15}N NMR Spectroscopy

Cross polarisation magic angle spinning (CPMAS) ^{15}N NMR spectroscopy has been extensively used for the characterisation of SON composition, because it allows the examination of soils or soil fractions without thermolytic or chemolytic pretreatment. The use of solid-state ^{15}N NMR for the analysis of soils is limited by its low sensitivity and the low N content of most soils. Therefore, ^{15}N NMR is often used to study the fate of ^{15}N-enriched materials in soils. Although resolution is limited, one can obtain an overview of the organic N structures present in the soil sample (Fig. 2.11). With appropriate acquisition parameters the determination of the signal intensities

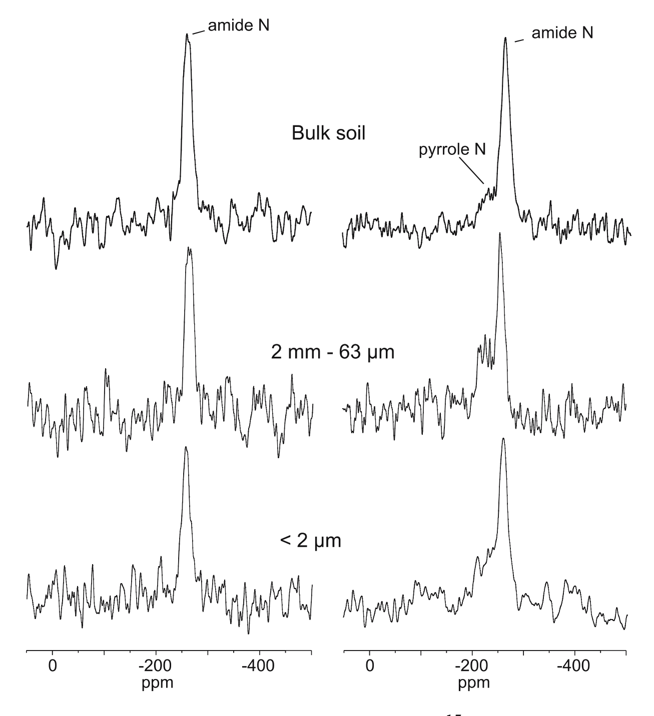


Fig. 2.11. Example for a solid-state CPMAS ^{15}N NMR spectrum of a soil not affected by fire and a fire-affected soil (H. Knicker, unpublished)

gives information about the average relative distribution of each identified functional group.

2.3.4 X-ray Absorption Near-Edge Structure Spectroscopy

Recently, synchrotron-based XANES has emerged as powerful tool for the speciation of various bioelements (e.g. C, S, Fe) in soils, and also for specific soil particles and microsites (Sparks 2001; Prietzel et al. 2003). XANES may also be a tool for exploring the complex chemistry of N and P in soils. Nitrogen K-edge XANES spectroscopy can be used to gain new insights into the speciation of the macromolecular N (Vairavamurthy and Wang 2002). This technique allows the identification and quantification of different species or species groups with different electronic oxidation states of the bioelement of interest without chemical degradation of the sample. The basic principle of XANES is that different species of a certain biolement in dependence of their oxidation states require different energies for core electron transitions to bound states or ejection into continuum and thus have different

resonance energies ('white lines'). The spectrum of a multi-species sample comprises several peaks with resonance maxima at different energies. After appropriate deconvolution of the spectrum into one or more edge steps and several single peaks, the different species of a bioelement in a sample can be identified by their specific peak maxima and quantified by the peak area.

2.4 Forms of Organic N in Soil Organic Matter

After entering the soil, material of biogenic origin experiences decay and microbial reworking. During these processes, the labile compounds are quickly mineralised to inorganic N forms, directly available for the production of new biomass. The more stable compounds and metabolic products accumulate to form the refractory organic pool of soils. Their N will be sequestered from the overall N cycle and therefore from bioproductivity over an extended time range.

The forms of organic N in soils are still a matter of debate. Solid-state CPMAS ^{15}N NMR spectroscopy suggests that almost all of the organic N is present in the form of amide bonds, i.e. as peptide or proteinaceous moieties (Knicker et al. 1993; Kögel-Knabner 1997), as shown in the example in Fig. 2.11. Amide N was also verified to be the dominant N form in humic substances and sediments by nitrogen K-edge XANES spectroscopy (Vairavamurthy and Wang 2002). Only in soils that had experienced vegetation fires were substantial amounts of N heterocyclics identified (Knicker 2004). In contrast, results from analytical pyrolysis indicate the presence of a large variety of heterocyclic aromatic N components (Schulten et al. 1997; Schulten and Schnitzer 1998). Most of the N heteroaromatics released with different pyrolysis techniques from soils are also produced from natural biological precursors, such as proteins, carbohydrates, chlorophyll, nucleic acids and alkaloids. They are at least in part artifacts produced during the pyrolysis procedure at high temperatures (Schulten and Schnitzer 1998; Kelemen et al. 2002).

Although the cell-wall polysaccharides of microorganisms are relatively easily decomposed, their basic units such as glucosamine, galactosamine or muramic acid are found in soil after hydrolysis (Bremner 1958; Stevenson and Cole 1999) or pyrolysis (van Bergen et al. 1998) and they accumulate during litter decomposition (Kögel and Bochter 1985; Coelho et al. 1997). Specific components of fungi and bacteria are thus used to estimate the contribution of microbial residues to SOM. Glucosamine is used as a marker for fungal cell walls and their residues, galactosamine, muramic acid and diaminopimelic acid for bacterial cell walls and residues (West et al. 1987; Kögel-Knabner 1993; Guggenberger and Haider 2002; Amelung

2003). Muramic acid was earlier proposed as an indicator for bacterial and cyanophyte biomass (Zelles 1988), but is supposed to be mainly present in dead cell-wall residues (Insam 2001).

Other N components, such as nucleic acids, are found in trace quantities only and require specialised techniques for extraction and identification (Nannipieri et al. 2003; Agnelli et al. 2004). From chemolytic techniques and analytical pyrolysis, total N in SOM is estimated to consist of about 40% proteinaceous materials (proteins, peptides, amino acids), 5–6% amino sugars, 35% heterocyclic N and 19% NH_3-N. The hydrolytic approach gives 20–30% acid-insoluble N, 20–35% NH_3-N, 30–45% amino acid N, 5–10% amino sugar N, and 10–20% hydrolysable unknown N (Stevenson and Cole 1999). The proportion of unknown SON that is not assignable to plant or microbial structures by Py-GC-MS is higher in the mineral soil compared to plant litter or forest floor layers (van Bergen et al. 1998).

An explanation for the high amide content in humified material may be the protection of proteinaceous material during humification via adsorption onto the mineral phase (Ladd et al. 1996; Chap. 4). The potential implication of the fine silt and clay fraction in protecting peptides from microbial or chemical degradation was recently demonstrated with solid-state CPMAS ^{15}N NMR spectra of the fine particle size fractions of different soils (Kögel-Knabner et al. 1996; Knicker et al. 1999, 2000). Most of their signal intensity is observed in the chemical shift region assigned to amide N. No signals of six-membered heterocyclic aromatic N or imines, which could confirm the catalytic involvement of clay minerals in the formation of Maillard products, were identified. Hydrolysis with 6 N HCl could only release less than 45% of this total N. Therefore, at least some of the organic N in these samples, identified as amide N, must be present in a form protected from microbial degradation and resistant to drastic chemical treatment. This resistance may explain to some degree the difficulties in identifying such structures with common wet analytical methods. Other mechanisms involve complexing with polyvalent cations (e.g. Fe^{3+} or Al^{3+}) or entrapment into micropores (Mayer 1994; Ladd et al. 1996; Sollins et al. 1996). A third possibility is the selective preservation of otherwise decomposable substrates in association with refractory biopolymers (Knicker et al. 1996; Zhang et al. 2000). Condensed tannins are supposed to sequester proteins from microbial degradation by complexation and precipitation (Maie et al. 2003). At the present stage of research, it is not possible to discriminate between or to favour one of the discussed mechanisms that may be responsible for the protection of peptide-like structures in mineral soils. It seems likely that they occur concomitantly. Their impact on SON stabilisation, thus, may be dependent on the respective chemical and physical conditions of the environment. Further studies have to reveal how these amide functional groups are protected.

The formation of heterocyclic aromatic N has been proposed as an important mechanism of N stabilisation during humification. Detailed descriptions of the proposed reactions, among others sugar-amine condensation, oxidative coupling, and the polyphenol theory, are found in Stevenson (1994), Bollag et al. (1998), and Jokic et al. (2004). Knicker et al. (2002) showed by ^{15}N NMR spectroscopic investigation of natural soils that heterocyclic aromatic N occurs only in environments where temperature and pressure have replaced the effect of microbial activity, i.e. during catagenesis or burning. Pyrrole- and indole-type compounds (Fig. 2.12) seem to be the major heterocyclic aromatic N compounds in soils resulting from fire events (Knicker and Skjemstad 2000; Knicker et al. 2005). These specific heteroaromatic N compounds may be formed during diagenetically or temperature-induced cyclisation and rearrangement. The frequent occurrence of vegetation fires and the use of fire-based management practices, such as the burning of brushwood and litter in woodlands, and of crop residues on agricultural soils, leads to the formation of SON in these specific structures and may add substantially to the role of soils as an N sink.

Using nitrogen K-edge XANES spectroscopy, Vairavamurthy and Wang (2002) identified pyridinic N (Fig. 2.12) also as a significant component of the total N (similar to 20–30%), with a subfraction consisting of its oxidised derivatives. An unidentified form of highly oxidised N was also present, but mainly in sediment samples. The authors concluded that amide N represents residues of original, biochemical molecules, but pyridinic N probably is generated abiotically. They suggest that the abiotic formation of pyridinic N sets in during the early stages of organic matter transformation thereby stabilising organic N, although such processes generating heterocyclic structures might continue much longer.

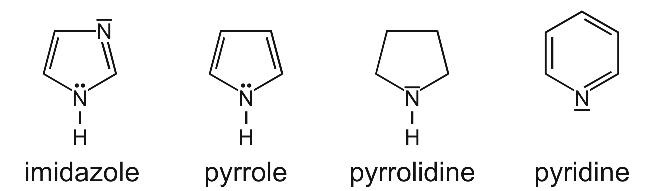


Fig. 2.12. Basic heterocyclic aromatic N structures supposed to be present in soil

2.5 Techniques To Analyse Organic P in Soils

The chemistry of organic P in soils is still obscure, again mainly due to a lack of appropriate methods to isolate and quantify individual compounds.

Various sequential extraction procedures are used to differentiate P pools in relation to soil development and bioavailability. Such extraction schemes give operationally defined pools, but do not give direct information on the structural composition of the various P compounds in soil, nor do they allow the identification of individual P components. ^{31}P NMR spectroscopy has been mainly used for alkaline or other soil extracts and thus only allows the identification of P functional groups in the extractable SOM.

2.5.1 Sequential Extraction and Separation

The conventional methods to differentiate organic and inorganic P components in soils basically rely on extraction with alkaline or acid reagents. They go back to the procedure originally developed by Chang and Jackson (1956), later further modified (Hedley et al. 1982; Kuo 1996). Many studies use NaOH, often in combination with chelating agents, such as EDTA, or resins. It is assumed that the chemical extractants sequentially remove discrete groups of P compounds. The inorganic P fraction in the extract is then determined by a colorimetric procedure and the amount of organic P is obtained by difference (Condron et al. 1985; Guggenberger et al. 1996; Sui et al. 1999; Koopmans et al. 2003). Another approach is to identify and quantify organic P forms with enzymatic mineralisation. The sample is incubated with specific P-releasing enzymes, such as alkaline or acid phosphatases, phytases or nucleases. The orthophosphate released is determined by colorimetric procedures, most often the molybdate blue method (Kuo 1996; Shand and Smith 1997; Hayes et al. 2000; He and Honeycutt 2001) and can be assigned to the organic binding form from which it was released.

2.5.2 ^{31}P NMR Spectroscopy

The magnetic properties and its 100% natural abundance make ^{31}P easy to be detected by NMR spectroscopy. The technique, introduced to soil analysis by Newman and Tate (1980), has been used for the examination of the chemical nature and transformations of P in a wide range of soil environments (Condron et al. 1997). The most common approach for ^{31}P NMR spectroscopy in soils is the analysis of NaOH extracts, sometimes in combination with chelating agents (Chelex, EDTA) in solution (Condron et al. 1985; Cade-Menun and Preston 1996). Cade-Menun et al. (2002) point out that the extractant strongly influences the yield of P extracted and also the P forms observed in the spectrum. Narrow lines are obtained in the NMR spectra, allowing identification of different chemical classes

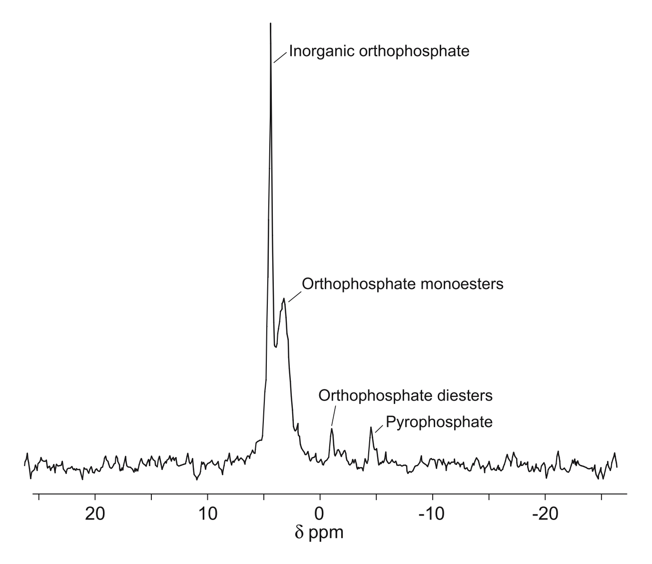


Fig. 2.13. Example for a ^{31}P-NMR spectrum obtained from an alkaline soil extract (from Condron et al. 1997)

such as orthophosphates, phosphate mono- and diesters, phosphonate, polyphosphates, or pyrophosphate (Fig. 2.13). ^{31}P NMR cannot clearly distinguish individual phosphate monoesters, such as the different inositol phosphates, sugar phosphates, and mononucleotides or orthophosphate diesters (phospholipids, nucleic acids, teichoic acids). Studies on the impact of soil type, climate, vegetation, cultivation and long-term fertiliser inputs showed a strong dominance of orthophosphate monoesters (Preston and Trofymow 2000; Cardoso et al. 2003; Turner et al. 2003a). ^{31}P NMR spectroscopy also shows the presence of other minor soil organic P species such as phosphonates and teichoic acids. Makarov et al. (2002) made a detailed study on the signal assignment in the diester region of ^{31}P NMR spectra. They were able to differentiate the signals for phospholipid P and teichoic acid P from DNA P and other unidentified P compounds that were previously attributed to phospholipids and nucleic acids. Turner et al. (2003b) could specifically identify signals from nucleic acids, microbial phospholipids and ATP/ADP. There is growing evidence that hydrolysis processes during the alkaline extraction procedure partially degrade orthophosphate diesters to monoesters (Makarov et al. 2002; Turner et al. 2003b). This seems to be especially relevant for RNA and some phospholipids, whereas orthophosphate monoesters and DNA seem to be more stable. These processes will also affect the relationship between monoester and diester P and lead to an underestimation of the original concentration of diester P in soils.

Solid-state ^{31}P NMR spectroscopy results in poorly resolved spectra, hindering any attempt to obtain detailed information (Condron et al. 1997). One possibility to overcome this problem was recently suggested to represent proton spin relaxation editing (PSRE). This technique takes advantage of differences in the relaxation behaviour of protons associated with the observed nuclei of different components of a mixture of two or more types of material. These types can be distinguished, if subspectra are obtained which only exploit signals of compounds with a specific proton spin lattice relaxation time constant. Applying this technique to a Pukaki soil from New Zealand, the broad line observed in the total spectrum was separated into three distinct peaks with different proton spin relaxation constants (Condron et al. 1997). The observed signals were assigned to inorganic ortho- and/or monophosphates, mono- and/or diesters and polyphosphates, respectively. Comparing the subspectra to those obtained from PSRE ^{13}C NMR experiments showed that the mono- and diesters were associated to strongly humified material. It was assumed that they are related to inert P species that survive through humification processes.

2.6 Forms of Organic P in Soils

Principal forms of organic P in soils are inositol phosphates, phospholipids, nucleic acids, but also their degradation products in various stages of decomposition. From the type of structures identified, organic forms of P in soils are orthophosphate monoesters $ROPO_3^{2-}$ (e.g. inositol phosphates, mononucleotides, sugar phosphates), orthophosphate diesters $R_1OR_2OPO_2^-$ (e.g. phospholipids, DNA and RNA, teichoic acids), and phosphonates, containing C–P bonds. Recovery of soil organic P follows the order inositol phosphates (10–50%) $\gg$ phospholipids (1–5%) > nucleic acids (0.2–2.5%). Metabolic phosphates, such as ATP, NADP, or sugar phosphates, are only found in trace quantities in soils (Stevenson and Cole 1999). The high abundance of inositol phosphates in soils compared to plants is ascribed to their ability to form insoluble complexes with polyvalent cations (Stevenson and Cole 1999; Turner et al. 2002). The inositol phosphates exist in soils as a family of six congeners of hexahydroxy cyclohexane (inositol), in various states of phosphorylation, i.e. between 1 and 6 phosphate ions are bound (Fig. 2.8). The most abundant identifiable organic P compound is phytate, i.e. hexaphosphate esters of inositol. They comprise up to 50% of total organic P in soils. Phytates carrying from one to five phosphate groups have also frequently been identified in soils and are supposed to be degradation products of inositol hexaphosphate. Stevenson and Cole (1999) point out that most of the inositol phosphates found in soil are supposed to be of

microbial rather than plant origin. As mentioned above, nucleic acids are highly susceptible to decomposition and not persistent in soil. This also explains why nucleic acid P makes up less than 1–3% of the total organic P identified in soils (Stevenson and Cole 1999).

2.7 Summary

Although much progress has been achieved in revealing more about the chemical structure of soil organic N and P and the mechanisms involved in their formation, we are still far from completely knowing the structures and understanding the formation and dynamics of these major soil organic matter components.

There is now consensus that a major part of the SON is present in the form of amide structures. Most of the identifiable N compounds in soils seem to be of microbial origin. The formation of heterocyclic aromatic N may be of less importance in SON stabilisation than formerly thought. Nonetheless, there is also evidence that such heterocyclic N compounds play a role in specific soil environments, especially such soils that have experienced vegetation fires or other similarly drastic conditions for SOM transformations.

Recent developments in the characterisation of organic P compounds, mostly in combination with ^{31}P NMR spectroscopy, have allowed a more refined identification of specific plant- and microbial-derived compounds. Most of these techniques are at present only applied to soil extracts and thus the nature of a major part of the organic P compounds in soils that are not extractable remains vague. The extraction conditions have to be chosen carefully to avoid degradation of ester P compounds.

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3 Nucleic Acid Extraction from Soil

Lars R. Bakken, Åsa Frostegård

3.1 Introduction

Methodological studies have a strong position in soil biology; many of us are method "freaks" willing to spend the best part of our lives developing new methods, or finding reasons to discard old ones. This preference is not entirely unjustifiable; soil is a non-transparent material in more than one sense, effectively hindering a direct observation of organisms and processes. Thus, our observations are very indirect (compared to other natural sciences); there is always a "method" (or several) involved which provides us with more or less biased and fragmentary impressions of the biological reality of interest. Progress in this field will be enhanced by creative inventions of new and partly complementary approaches. For each method invented, tested, and discarded, small pieces of facts about the life of soil microbes are provided. For more than two decades, soil ecologists have analysed nucleic acids in soils for various purposes (Trevors and Van Elsas 1995; Wellington et al. 2003). The tools have developed rapidly, allowing analyses of community composition, phylogenetic and functional groups, specific species/strains and functional genes (Nannipieri et al. 2003). Most work has been done on DNA, but more recent developments include RNA, which provides information about the active part of the microbial community (Moran et al. 1993; Felske and Akkermans 1998; Duineveld et al. 2001) and gene expression (Bürgmann et al. 2003). Prior to any such analyses, the soil material must be removed; the nucleic acids or the microbial cells must be extracted from the soil particles and humic substances which will otherwise obscure almost any observation. The number of protocols invented is daunting, reviewed several times over the last two decades (Trevors 1992; Saano et al. 1995; Torsvik et al. 1995; Van Elsas and Smalla 1995; Stapleton et al. 1998; Roose-Amsaleg et al. 2001; Gabor et al. 2003; Robe et al. 2003). Figure 3.1 illustrates the essential steps taken in different protocols to

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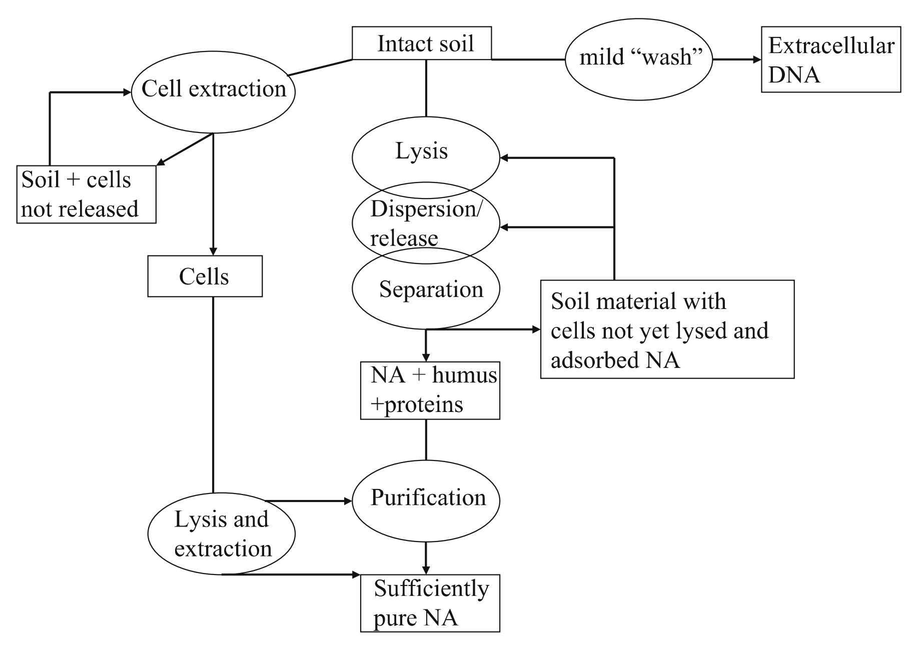


Fig. 3.1. Flow charts for direct extraction of nucleic acids (NA) or extraction and purification of cells prior to NA extraction. The most common operations (*in ellipses*) and their intended products (*in boxes*) are shown

achieve such extraction. The protocols can broadly be divided in two main categories: (1) direct extraction of nucleic acids (NA) from soil, sometimes preceded by a mild washing to remove extractable extracellular DNA, and (2) cell extraction and purification prior to NA extraction/analysis.

From time to time, critical evaluations have revealed that cell lysis and NA extraction represent the Achilles' heel of molecular methods in soil biology (Frostegård et al. 1999; Miller et al. 1999; Westergaard et al. 2001). It has been shown that the DNA material extracted from soil is not representative for the entire soil community in question, for a number of reasons; presence of extracellular NA (mostly DNA), biased cell lysis, losses due to shearing and enzymatic degradation, sorption to colloids, and losses during purification. Further, it has been a recurring experience that humic substances, which contaminate the extracted NA, may inhibit enzymes that are essential for the analysis of NA (Stach et al. 2001).

Finally, the success of protocols may be limited to specific target organisms/genes and/or specific soils. It appears unlikely, therefore, that a single consensus protocol should emerge to replace the plethora currently in use. It is more likely that protocols will continue to be designed, ad hoc. In designing such protocols, it is crucial to have an understanding of the

mechanisms involved in the various steps, and the types of bias they may create. We hope that the present chapter will provide an updated knowledge of this kind. A leitmotif could be Leonard Cohen's "there is a crack in everything, that's how the light comes in"; a biased method is problematic (cracks) until we know the extent and the reasons for the bias. With that knowledge, experiments can be designed to exploit the bias to unravel new phenomena (... how the light comes in).

3.2 Lysis and Extraction

All protocols for NA extraction from soil microorganisms include treatments meant to achieve lysis of the cells (Robe et al. 2003) since large molecules, like whole genomes and ribosomes, are unlikely to be released if the cell sacculus is intact (mRNA, however, is not necessarily retained by the cell sacculus if the membrane is destroyed). Most protocols combine chemical and enzymatic means with physical means to achieve cell rupture and extraction, either in sequential steps or in a single operation.

3.2.1 Cell Rupture Depends on Cell Type and Growth

Physical means to rupture cells are generally more efficient on Gram-negative than Gram-positive bacteria, due to the thicker peptidoglycan layer of the latter. The strength of the peptidoglycan layer is not only dependent on its thickness, but also on the degree of the peptide cross-links (Pisabaro et al. 1985). The number of cross-links can vary between species, but for each species it also depends on growth rate. Growth will necessarily imply that the wall must be weakened, to allow it to expand (Koch 2001). The degree of cross-linking of the peptidoglycan layer of *E. coli* is higher in slow growing than in rapidly growing cells, and maximum in stationary stage cells (Pisabaro et al. 1985), and stationary stage *E. coli* and *B. subtilis* were found to be much more resistant against sonication than cells from an exponentially growing culture (Lindahl and Bakken 1995). Similarly, fast growing *Pseudomonas aeruginosa* were more susceptible to EDTA+lysozyme treatment than stationary stage cells (Watt and Clarke 1994). The morphology and cell size of bacteria are also of importance (Geciova et al. 2002), rods are easier to disrupt by impingement than cocci, and large cells easier than small ones (Kelemen and Sharpe 1979). Fungal cells are at least as variable as bacteria regarding wall thickness and strength, and not the least size and morphology. Similar to bacteria, the

thickness and thus mechanical strength of fungal walls appear to be inversely related to growth rate, reaching a maximum at stationary stage (Smith et al. 2000). Melanin accumulation in aging fungal walls may confer some increased strength as well as a better resistance against enzymatic degradation (Kuo and Alexander 1967). Finally, endospores of bacteria and fungal spores are generally much more resistant against rupture than the vegetative cells (Kuske et al. 1998). Protozoa have extremely resistant resting stages compared to their vegetative cells, and as a result harsh methods such as bead beating have been recommended for extraction of their DNA (Fedorko et al. 2001; Fredslund et al. 2001).

In conclusion, the composition of the extracted DNA may be severely biased; metabolically active and/or large cells will tend to be over-represented compared to stationary stage and/or small coccoid cells. On the other hand, extending mechanical cell rupturing to include the toughest cells may create the opposite bias: the DNA from the most fragile cells may be "lost" by shearing (see below). The severity of this dilemma clearly depends on the minimum size of the DNA molecules for the application planned. If DNA is extracted for polymerase chain reaction (PCR)-based analysis of small gene segments, the shearing may not be a serious problem. At the other extreme, for the creation of metagenome libraries, very large pieces of DNA are desired.

It should also be mentioned that the mechanisms of cell rupture are not obvious, and this could hypothetically have consequences for the bias (discussed below). Kleinig and Middelberg (1998) claim that inertial forces (internal pressure gradients caused by sphere acceleration) may be important in high-pressure cell homogenisers (and in sonicators) for large cells (yeasts), whereas viscous forces are more important for smaller cells (low Reynold's number; Purcell 1977). Compression of materials, as in tabletting, is thought to kill cells through viscous shear rather than by heat (Blair et al. 1991); thus cell rupture/lysis is the cause of observed death. Similar mechanisms are probably involved in soil grinding in mortars (Frostegård et al. 1999) and in mortar grinding of frozen soil or cell suspensions (Zhou et al. 1996; Hurt et al. 2001), although brittle fracturing across the frozen cells is probably also of importance in that case. Finally, cell rupture by deformation when pressed between colliding solid surfaces (Smith et al. 2000) is probably the most important factor in bead beating.

3.2.2 Bead Beating, Efficiency and Bias

Bead beating has been a popular component of cell lysis protocols. A soil slurry (or a suspension of extracted cells) is mixed with small spheres (most

commonly 0.1 or 0.2 mm spheres, sometimes mixed with larger beads) and shaken at high frequency. Bead beating exploits the collision between glass spheres to break the cells; thus deformation is probably the main cause of cell disruption, although viscous shear may hypothetically be important at high speeds.

Recognised critical physical parameters in bead beating are the proportions between soil, water and glass beads, the frequency of beating, and the length of the treatment (Bürgmann et al. 2001). Efficient lysis can obviously be achieved by extending the treatment over several minutes at the cost of shearing the released DNA (viscous shear of DNA is negligible as long as the DNA is packed within a cell sacculus, but significant when the DNA is freely suspended after cell rupture). The choice of bead beating time is thus a choice between pest and cholera: short bead beating times will only lyse the fragile cells while more robust cells may be lysed by prolonged beating, at the cost of shearing the DNA from the fragile ones. Bürgmann et al. (2001) conducted a thorough comparison of various versions of bead beating for PCR. Even bead types were compared: a mixture of different beads (0.1, 1.4 mm and a single 4 mm sphere) performed better than beads of equal size; but shearing was aggravated. A plateau in DNA amounts was reached by a treatment of 6 m s^{-1} (6,300 rpm) for 45 s; harsher treatments yielded very little extra. Neither did repeated treatment (except for lower intensity procedures).

The kinetics of cell lysis and DNA shearing after bead beating or grinding has been systematically investigated by some researchers (Van Elsas et al. 1997; Frostegård et al. 1999; Bürgmann et al. 2001) while many others appear to use shear guesswork when deciding their bead beating times, and they appear to have made widely different guesses, as judged from the large differences between protocols (Table 3.1). Strict comparisons are difficult, however, since few authors report the intensity of the bead beating properly. There are at least two parameters which are of importance: the frequency and the amplitude, which together determine the frequency and energy of collisions between the beads. Some authors simply report "full speed", some report frequency and some speed (m s^{-1}), neither is adequate. In addition, the nature of the movements (rotations of reciprocal) may have some influence on the energy of the collision; such information is seldom reported, neither by the producer of the equipment nor by the authors of scientific papers.

We have made a rough calculation of the relationship between cell size and cell rupture by deformation based on the following assumptions:

1. Deformation of the glass beads at collision is negligible.
2. Hence, each collision will rupture cells within a liquid volume which can be calculated by straightforward geometry, as a function of the bead

Table 3.1. Bead beating protocols for cell rupture to extract NA

Reference	Device/ Producer	Speed	Time (min)	Beads/ liquid/soil[a]	Size (mm)	Solutions[b]	
Smalla et al. 1993	Braun homogenizer	4000 Upm	5–10	1:2:2	0.17–0.18	Phosphate	120 mM (pH = 8)
						Lysozyme	5 mg ml^{-1}
Briglia et al. 1996	Bead beater B Braun M	No report	1.5–4.5	2:1:1	0.09–0.11	Distilled water	
Moré et al. 1994	Biospec, Bartlesville, Oklahoma	"full speed"	5–10	2:1.5:0.5	0.1	Phosphate	100 mM (pH = 8)
Kuske et al. 1998	BX-4 from Biospec	5,000 rpm	3	2:1[c]	0.7 and 1.2 (equal amounts)	Tris-HCl	50 mM
						Na-EDTA	20 mM
						NaCl	100 mM
						SDS	1%
Howeler et al. 2003	Biospec	3,300 rpm	5	1.5:0.9:0.4	0.1	Tris-HCl	160 mM
						Phosphate	33 mM (pH = 8)
						NaCl	33 mM
						SDS	3%
						Chloroform	0.32 ml ml^{-1}
						Isoamylalcohol	0.013 ml ml^{-1}
Borneman et al. 1996	FastPrep FP120	5 m s^{-1}	0.5	nr[d]	nr[d]	FastDNA spin kit following manufacturers' instructions	
Cullen and Hirsch 1998	Microdis-membrator II, Braun, Germany	amplitude 5 mm	5	1:3:1	0.5, 2 and 3 (equal amounts)	Phosphate	120 mM (pH = 8)
						SDS	1%

[a] Weight ratios between liquid, beads and soil dry weight.
[b] The enzyme treatment precedes the bead beating.
[c] A cell suspension, not soil. The liquid volume includes the cell suspension added.
[d] FastDNA tube containing a matrix "designed to lyse most cell types" (Bio 101, Catalog no. 6530–401).

diameter and the critical deformation, **Dc**, which is the distance between surfaces below which the cell will burst.

3. The calculated "rupture volume", $\mathbf{v}_r$ (the volume of liquid in which cells will be ruptured by deformation), is necessarily a function of cell size and physical characteristics of the wall.
4. The rupture rate (**Rs** = fraction of cells ruptured by a single stroke) is then a function of the probability of a single cell to be in one of the "rupture volumes", which again is a function of total liquid volume ($\mathbf{V}_{tot}$) and the total number of beads ($\mathbf{N}_b$), and the assumed number of successful collisions per bead for each stroke ($\mathbf{C}_b$): $\mathbf{Rs = v_r * N_b * C_b / V_{tot}}$.
5. The rupture rate has thus been calculated for beads with a diameter of 100 μm (which is the most frequently used), $V_{tot} = 1$ (ml per g of beads), $C_b = 4$. The calculation has been done for cells with three critical deformation distances, **Dc**: 0.3, 0.5 and 1 μm. The results are shown in Fig. 3.2.

The results are in reasonable agreement with the observations of Moré et al. (1994): assuming that their beat speed was 3000 rpm, a 5 min beating equals 15,000 strokes in Fig. 3.2. It illustrates the dilemma when choosing

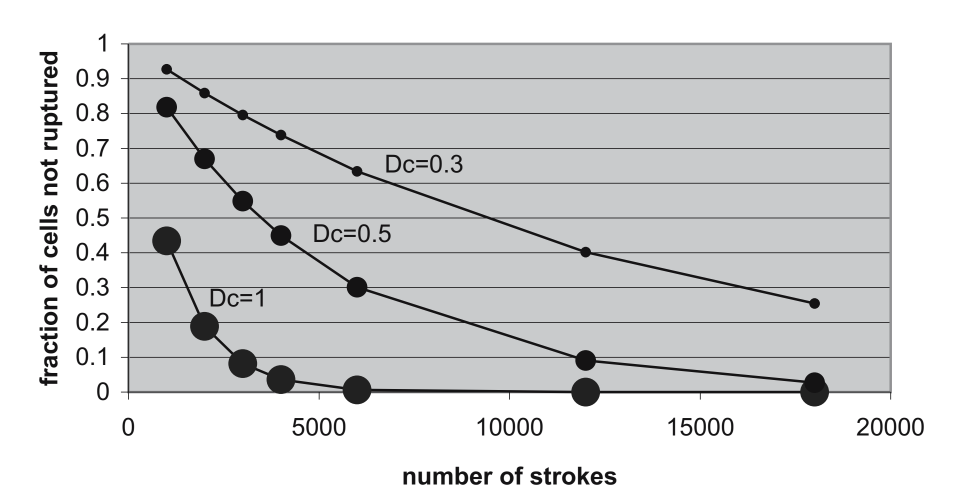


Fig. 3.2. Estimated cell rupture in a bead beater with 1 g liquid per g of 0.1 mm beads, as a function of the critical deformation (Dc, μm), i.e. the distance between surfaces below which the cell will burst. The figure shows the fraction of the initial cell numbers (= 1 for each group) remaining intact (unruptured), plotted against the number of strokes. The calculated rupture rate (stroke^{-1}) depends on bead size. For larger beads, for instance 0.17 mm, as used by Smalla et al. (1993), the calculated rates would be around 20% of the rates calculated here. In other words, their treatment at 4000 rpm for 10 min would end up at 40,000/5 = 8,000 strokes in the present figure

bead beating intensity (time or speed). A nearly complete rupturing of large/brittle cells can probably be achieved within half a minute at high speed (as in Borneman et al. 1996).

Bead beating has also been used for lysis of fungi in soil samples. For example, after addition of *Aspergillus fumigatus* to soil, higher amounts of DNA were recovered after bead beating following the protocol of Cullen and Hirsch (1998) than after grinding in liquid nitrogen (Kabir et al. 2003) while, in another investigation, liquid nitrogen grinding gave similar recoveries and was therefore preferred to the more time-consuming method based on bead beating (Van Elsas et al. 2000). Although the efficiency of different lysis treatments on indigenous soil fungi has not been well studied, clones containing 18S rDNA sequences from the main fungal phyla (Ascomycetes, Basidiomycetes, Zygomycetes and Chytridiomycetes) were obtained after bead beating and direct DNA extraction (Anderson et al. 2003; Gomes et al. 2003).

3.2.3 Grinding

Various versions of grinding have been used to rupture cells, either by grinding dry soil (Frostegård et al. 1999) or a frozen (liquid nitrogen) soil or cell suspension (Zhou et al. 1996). The protocol developed by the French group (Frostegård et al. 1999; Courtois et al. 2001) includes a machine mortar grinding of air dry soil, partly to rupture cells mechanically, partly to ensure access of the enzymes (lysozyme and achromopeptidase) used in the next step of the protocol. The grinding of dry soil, whereby the soil mineral particles are completely pulverised, probably ruptures cells by viscous shear as well as by deformation between colliding soil particles. The rupturing efficiency was only moderate; direct microscopic counts revealed that more than 50% of the cells remained structurally intact (detectable by acridine orange direct counts) after grinding (Frostegård et al. 1999). The bias with respect to cell size was not investigated. It appears likely that small cells survive better than large ones but the calculation of this bias is not as straightforward as for the bead beating (above).

The grinding of cells in frozen condition (most often in a hand mortar with liquid nitrogen as the freezing agent) will rupture the cells by brittle shearing as in freeze-fracture/freeze etch techniques for electron microscopy preparations. The method is not uncommon for NA extraction from fungi (Nazar et al. 1996; Kabir et al. 2003) but less frequently used when bacteria are targeted. Zhou et al. (1996) examined its effect on various Gram-positive species (cultured); cell pellets were mixed with sterile sand and liquid nitrogen and ground (hand mortar, unspecified intensity and

length of time), resulting in substantial (2–6 times) increase in extracted DNA compared to freeze/thaw (liquid nitrogen/microwave heating till brief boiling), both treatments in 2% sodium dodecyl sulfate (SDS). In contrast, Kabir et al. (2003) experienced severe shearing by a similar freeze-grinding of soil seeded with bacteria. To our knowledge, there exists no direct microscopic investigation of freeze-grinding on cell rupturing, neither on the total nor on the size-dependent bias. In theory, the size-bias of brittle fracturing of frozen cells should be similar to the bias in bead beating, for shear geometric reasons (a large cell is more likely to be hit by a fracture plane than a small cell). Consequently, the method is likely to be more efficient for fungi than for bacteria.

3.2.4 Freeze/Thaw

Freeze/thaw is also a method believed to rupture cells, used in various combinations with SDS and enzymes. Ice crystal formation during freezing is well known to cause cell rupture (hence glycerol is added when cultures are preserved by freezing) but the effect on microbial cells in soil appears moderate. Smalla et al. (1993) found bead beating to be substantially more efficient than freeze/thaw (both conducted after lysozyme and cold SDS treatment). Both Kauffmann et al. (2004) and Zhou et al. (1996) concluded that freeze/thaw in combination with SDS may be sufficient to lyse Gram-negative cells but not Gram-positive ones. The main effect of the freeze/thaw may be to release DNA from already lysed cells (Tsai and Olson 1991). Thus freeze/thaw treatment has often been used subsequent to lysozyme treatment and in combination with SDS (Rochelle et al. 1992; Lee et al. 1996), or subsequent to bead beating with SDS (Moré et al. 1994; Ogram et al. 1995).

3.2.5 Enzymatic Lysis

Enzymes are often used to lyse cells, either alone or in combination with chemicals and physical means, or simply to permeabilise the cells to allow transport of materials in and out of the intact cell sacculus (for microscopic analyses such as fluorescence in situ hybridisation, FISH). As a rule, enzymes are selective which the detailed review below will show.

Lysozyme, by far the most commonly used enzyme, dissolves bacterial cell walls by hydrolysing the β-1–4 glycoside bonds in the peptidoglycan in eubacterial walls. The outer membrane of Gram-negative bacteria is

a barrier which may protect the cell wall against lysozyme, but the protection appears to be only partial and variable among strains (Masschalck et al. 2001). Archebacteria are resistant to lysozyme since their walls are made up of other materials than peptidoglycan. However, even among the Gram-positive bacteria, there are groups (e.g. Actinomyces) which are relatively resistant to lysozyme (Barsotti et al. 1988). Permeation of the cell wall of Actinobacteria was shown to be only partial with lysozyme, and the combination of lysozyme and achromopeptidase was much more efficient (permeation of fixed cells for FISH; Sekar et al. 2003). Lysozyme is inhibited by SDS (Smith and Stoker 1949) which is one good reason to use lysozyme and SDS in sequence rather than in one single step. Lysozyme has also been used in protocols to extract fungal DNA (Claassen et al. 1996; Landeweert et al. 2003) which is not as absurd as it may sound: lysozyme has been demonstrated to have chitinolytic activity (Pooart et al. 2004; Takeshita et al. 2004), probably by having two different active sites (Takeshita et al. 2003).

Achromopeptidase is a protease extracted from *Achromobacter lyticus* which attacks the peptidoglycan layer of bacteria by cleaving its peptide cross bonds. Commercial bacteriolytic achromopeptidase reagents may contain both alpha- and beta-lytic proteases and possibly others (Li et al. 2000). The alpha protease attacks the polysaccharide-peptide bond and the D-Ala-Gly and Gly-Glu bonds (Li et al. 1997); the beta protease cleaves the glycine-X peptide bond (Li et al. 1998). Achromopeptidase is characterised by a very high proteolytic activity, broad pH range, and a high stability to denaturation. For instance, achromopeptidase retains high activity in the presence of 0.1% SDS and 5 M urea (Tsunasawa et al. 1989). Inhibition by chelating agents (EDTA and phenanthroline) has been observed, however, suggesting that achromopeptidases are metalloproteins (Li et al. 2000). It is probably smart, therefore, to avoid EDTA (or other chelating agents) as an additive in achromopeptidase cocktails. Although achromopeptidase appears rather unspecific, the enzyme (in combination with lysozyme) was shown to leave certain Gram-positive cells (belonging to the genus *Micromonospora*) intact while others were efficiently lysed (Frostegård et al. 1999).

Other enzymes are sometimes used in combination with the peptidoglycan-targeting enzymes, in order to help the latter to gain access to the peptidoglycan layers. Thus, protein-rich extracellular material has been targeted with proteinase K or mutanolysin. Lipase and mutanolysin were used in combination to lyse spores of *Bacillus anthracis* (Sjöstedt et al. 1997). The reason for using lipase is not clear. Mutanolysin is a lytic enzyme with a high specificity for lysing certain Gram-positive bacteria but not Gram-negative ones (Yukogawa et al. 1974; Raddadi et al. 2004).

Novozym 234 (Novo, Denmark) is a mixture of chitinolytic enzymes from *Trichoderma harzianum* which efficiently lyses fungal cells, and has

been used to produce fungal protoplasts (Robinson and Deacon 2001) and to extract DNA from cultures (Varma and Kwonchung 1991) and soil (Porteous and Armstrong 1991). The mixture contains DNase which will reduce the DNA yield unless inhibited (Varma and Kwonchung 1991).

3.2.6 Chemical Agents

Chemicals include a range of compounds which are used to permeabilise and thus to enhance enzymatic attacks and/or physical rupture (Nandakumar et al. 2000). The outer membrane of Gram-negative bacteria protects the peptidoglycan and can be destroyed/permeabilised by adding EDTA (Watt and Clarke 1994), detergents like SDS and Triton X-100 (Andrews and Asenjo 1987), or more specific compounds such as polymyxin B (Katz et al. 2003). The specificity (at strain or species level) for some of these compounds is possibly a problem. It is interesting to note that not only may they be strain-specific, the effects may also depend on the growth rate of the cells. Watt and Clarke (1994) observed that EDTA effects (in concert with lysozyme) depended on the growth rate of the cultures; stationary stage cells were much more resistant than actively growing ones. As mentioned earlier, the difference may have been due to differences in the cross binding in the peptidoglycan layer but may also be due to membrane changes. Many protocols for DNA extraction include a "hot SDS lysis", in which a high SDS concentration (1–4%) is used and temperature is either kept constantly high (65 °C; Zhou et al. 1996) or alternating between frozen (liquid nitrogen) and warm (50 °C; Clegg et al. 1997).

3.2.7 Extraction for Metagenome Libraries

To extract bacterial DNA for the creation of clone libraries (metagenome projects; Rondon et al. 2000; Lorenz and Schleper 2002), it is desirable to achieve high molecular weight and unbiased DNA (Lorenz and Schleper 2002; Berry et al. 2003). The necessity of large DNA inserts is particularly important when using vectors which can carry large (100 kb) inserts (BAC, Cosmid) which are used to construct libraries suitable for sequence homology-based screening. Thus, Ginolhac et al. (2004) use a DNA extraction protocol which ensures a minimum of physical DNA shearing; cells are first extracted from the soil (on Nycodenz), then mounted in an agarose gel plug prior to lysis (by lysozyme and achromopeptidase) and extraction (100 mM EDTA, 1% lauryl sarcosyl, proteinase K).

However, even for libraries used for functional screening (using plasmids or lambda vectors with strong promoters), the size of the insert will affect the chances of expression (Gabor et al. 2004). Lee et al. (2004) used the > 25 kb size fraction of soil DNA (extracted by the freeze/thaw enzymatic method; Zhou et al. 1996) to construct a metagenome library (in *Escherichia coli* with a cosmid vector), which was screened for lipolytic activity. Gabor et al. (2003) used somewhat smaller inserts (4–6 kb, prepared by mechanical shearing of soil DNA) inserted with the pZero-2 vector (Invitrogen) to create a gene bank (*E. coli*) screened for novel amidases.

Based on the foregoing discussion of cell lysis, it appears practically impossible to achieve an unbiased DNA extract from entire communities without shearing the DNA. The conflict between efficient lysis and minimum shearing was demonstrated by Kauffmann et al. (2004) who compared two extraction methods, the method of Zhou et al. (1996; freeze thaw, proteinase K and SDS) and the bead beating procedure of Moré et al. (1994; bead beating, SDS), for extraction of DNA from a Gram-negative (*Pseudomonas* LU2023) and two Gram-positive species (Arthrobacter LU9144, *Rhodococcus* LU9002). Pseudomonas DNA was efficiently extracted with both methods but the bead beating resulted in substantially more shearing of its DNA. In contrast, bead beating was the only method able to lyse and extract DNA from the Gram-positive cells. By using the two methods in sequence (bead beating extraction of cell remaining after Zhou extraction), they achieved two "biased" DNA extracts, one dominated by high molecular weight DNA from *Pseudomonas* (after Zhou extraction), and one dominated by DNA from the Gram-positive cells (bead beating extraction of the cells remaining after the Zhou extraction).

3.3 Purification

The crude DNA extract derived from soil organisms generally contains humic substances and other compounds which inhibit enzymes such as polymerases and restriction endonucleases (Tebbe and Vahjen 1993; Wilson 1997). Naturally, DNA obtained after direct in situ lysis is more contaminated than DNA derived from cells that have been separated from the soil before lysis. The contamination with inhibiting substances in direct in situ lysis can be reduced by prewashing of the soil (buffer and EDTA) prior to lysis (Fortin et al. 2004), but purification of the extracted DNA is often necessary. For this, a number of strategies have been adopted, thoroughly reviewed by Robe et al. (2003). Some are time-consuming and others are quick and easy to perform, some are inexpensive and others relatively expensive. Often, several methods are combined to obtain satis-

factory results. The choice of purification method depends not only on soil type and hence the type of contaminants present, but also on the goal of the investigation. Moreover, it should be kept in mind that increasing the number of purification steps will probably lead to increased losses of target NA.

Inhibitors often originate from the samples, e.g. humic and fulvic acids from soil DNA extracts, but care should also be taken during the whole process of sample handling since dust, pollen, glove powder and numerous other exogenous contaminants may have inhibitory effects (Wilson 1997). Mechanisms for inhibition are not well understood; it is likely that different chemical and physical factors interfere in the interaction between DNA and enzymes, for example, by unspecific blocking of target or primer DNA thus making it unavailable for polymerase (Wilson 1997). It appears also that humic substances may reduce the specificity of primers used in PCR (Stach et al. 2001). In the case of hybridisation, saturation of the membrane by the contaminants appears to be the problem or, alternatively, interaction between contaminant and target NA (Alm et al. 2000). In the following we list the major contaminants of soil-derived NA extracts known today, and give brief descriptions of the most common ways to remove them.

Humic acids and other phenolic compounds are major contaminants of soil DNA. Nucleic and humic acids have similar characteristics, and are therefore difficult to separate. A number of strategies have been tried (Wilson 1997; Roose-Amsaleg et al. 2001), many dependent either on differential binding to a polymeric matrix or fractionation based on size differences. Polyvinylpolypyrrolidone (PVPP; Holben et al. 1988; Picard et al. 1992; Frostegård et al. 1999) and hexadecyltrimethylammonium bromide (CTAB; Porteous and Armstrong 1991; Saano et al. 1995; Fortin et al. 2004) are often added to the extraction buffer since they form complexes with humic acids. Selective precipitation of humus can also be achieved by $AlNH_4(SO_4)_2$ (Braid et al. 2003). Very often columns are used through which the crude DNA extract is passed. The columns can be packed with PVPP (Kabir et al. 2003), but generally prepacked, commercially available columns are used, such as Wizard columns (Promega, Madison, WI, USA; Van Elsas and Smalla 1995; Harry et al. 1999) or Elutip-d columns (Schleicher and Schuell; Picard et al. 1992; Frostegård et al. 1999) all of which are based on binding to a polymeric matrix. Alternatively, gel filtration resins can be used, such as Sepharose 4B, Sephadex G-200, G-75 or G-50 (Jackson et al. 1997; Cullen and Hirsch 1998; LaMontagne et al. 2002) or Sephacryl S-400 (Frostegård et al. 1999) which are based on size fractionation.

Proteins, polysaccharides and non-target nucleic acids are other contaminants that can cause severe inhibition of enzymatic reactions (Tebbe and Vahjen 1993; Lantz et al. 1997; Wilson 1997). Proteins are often digested by the addition of proteinase K (Porteous and Armstrong 1991), a non-specific

serine protease, or (Jacobsen and Rasmussen 1992), which is a mixture of endo- and exo-proteinases. These proteases are active at the pH and salt levels generally used, and remain stable in the presence of SDS. Another way to remove proteins which is often employed is phenol/chloroform extraction (Sambrook et al. 2004), and yet another is precipitation in potassium acetate (1.3 M final conc.; Smalla et al. 1993) and/or cesium chloride (CsCl, 5.3 M final conc.; Van Elsas and Smalla 1995). Polysaccharides are claimed to cause problems and can be removed by polyethylene glycol (PEG) precipitation (Porteous and Armstrong 1991). DNA extracts are often treated with RNase to remove contaminating RNA and, similarly, RNA extracts are treated with DNase.

Alternative ways of separating NA from impurities are purification by CsCl gradient centrifugation (Courtois et al. 2001) or gel electrophoresis (Rasmussen and Sørensen 2001; LaMontagne et al. 2002). Moreover, a range of DNA extraction kits are marketed which contain purifying agents or provide purification step(s); e.g. FastDNA spin sample kit (for soil; BIO101, La Jolla, CA, USA) where the Geneclean purification uses Glassmilk which contains ground SiO_2; or the UltraClean Soil DNA kit (MoBio Laboratories Inc., Solana Beach, CA, USA). When specific NA sequences are targeted, magnetic beads can be used to "fish" for these in a complex DNA extract (Jacobsen 1995).

3.4 RNA Extraction

The general principles for DNA and RNA extraction are the same, but the extraction of RNA requires special precautions to inhibit RNase activity, in particular if mRNA is the target (Ogram et al. 1995; Ye et al. 2001). Many different RNases have been reported from various microorganisms (Nicholson 1999), and the catalytic mechanisms of all these are not yet fully understood. DNases require metal ions to be active, and thus can be inhibited by chelating agents such as EDTA. The activity of Rnase A (the most well known of the RNases) instead depends on histidine residues which constitute the enzyme's active site and react with the 2′ hydroxyl groups, situated close to the phosphodiester linkages in RNA, resulting in cleavage of the phosphodiester bond of the RNA backbone.

RNases exist in most organisms, and special laboratory procedures are needed to keep all solutions and equipment free from these enzymes (Blumberg 1987). Ideally, a laboratory room, or an area in a room, should be dedicated for RNA work. Only sterile disposables should be used, and gloves should always be worn to avoid contamination of the solutions/equipment by the introduction of RNases, or bacteria, from the skin. Apart from

these "common sense" rules, strategies to suppress RNases include denaturation of the enzymes, inactivation, or exclusion by ultrafiltration (http://www.millipore.com). Many RNases can withstand autoclaving and also long periods of boiling, and there are also indications that RNases might renature after autoclaving. High temperature over long time is needed to permanently denature RNases, e.g. all glassware should be baked at 180 °C for several hours. The most common way of suppressing RNase activity in solutions is by treatment with diethylpyrocarbonate (DECP). The molecular mechanism behind the inactivation is not known in detail for all RNases, but in the case of RNase A the DECP molecules react covalently with the histidine 12 residues, resulting in a permanent inactivation of the RNase enzyme. After treatment with DECP (often 0.1% conc.) the solution should be autoclaved to eliminate excess DECP. During autoclaving, the DECP molecule is degraded to ethanol and CO_2, of which small amounts will remain in the solution as contaminants. An alternative to this chemical treatment is therefore ultrafiltration, which efficiently removes RNase from solutions.

3.5 Cell Extraction

The extraction of intact cells prior to cell lysis and NA extraction is attractive for a number of reasons. Contamination by interfering substances (humus) can be practically eliminated. Extracellular DNA (or RNA) is also eliminated, at least with the density-based protocols (see below). Cell extraction will also eliminate the potential problem with losses of NA by sorption to soil colloids, which appears to be the reason that some protocols are a complete failure for some particular soils. The main argument against cell extraction is that it is incomplete (normal yield is between 10 and 20% of all bacterial cells), hence potentially biased. Another reason for not choosing cell extraction has been that it is time-consuming and complicated. This was true for old protocols but great simplification was achieved by using a single-step dispersion/density-gradient centrifugation (Bakken and Lindahl 1995). Further improvements have been implemented to provide cell samples for flow cytometric investigation of reporter organisms (Unge et al. 1999).

In contrast to the plethora of protocols for NA extraction, only a few variants of the cell extraction have been invented. The principal scheme is dispersion of soil and subsequent centrifugation to separate soil particles from cells, either based on differences in sedimentation rates or buoyant density.

3.5.1 Dispersion

Physical dispersion can be achieved by using any blender, as long as it does not seriously rupture the cells in question. Which most blenders will, however, if given enough time: a 2% decay rate per minute of homogenisation was recorded for *E. coli*, *Bacillus subtilis* (vegetative cells) and methanotrophic bacteria, when homogenising 200 ml suspension in a 1,000 ml container in a Waring blender at full speed (18,000 rpm) with cooling (Bakken and Lindahl 1995). The respiration rate in a soil homogenate showed a similar (2% min^{-1}) decay rate (Bakken and Lindahl 1995). It appears, therefore, that the procedure of 3 min blending (Waring blender), devised by Fægri et al. (1977), was a reasonable compromise between maximising the dispersion at the cost of a marginal (6%) cell rupture of fragile cells. It is by far the most common way of soil dispersion for cell extraction, and appears still recommendable as the default procedure. Milder dispersion procedures have been used for separating reporter bacteria by density-gradient centrifugation (for flow cytometry), either by vortexing for 1 min (Unge et al. 1999) or mild sonication (Burmølle et al. 2003). It is a good idea since such cells are often fragile. It may also be used to extract the most metabolically active fraction of indigenous soil bacteria (Lindahl et al. 1996).

Chemical dispersion agents have been discussed by Bakken and Lindahl (1995) at length. Detergents are thought to enhance cell release by dissolving extracellular materials but may also damage cells. Other additives have been tried, such as PEG (Herron and Wellington 1990) and PVPP (Steffan et al. 1988). The purpose with these two compounds is primarily to precipitate humus and other contaminations. Not surprisingly, therefore, PVPP additions resulted in some decrease in the yield of indigenous soil bacteria (Steffan et al. 1988). In contrast, PVPP addition improved the extraction of Pseudomonas cells which had recently been added to the soil (Unge et al. 1999). The contrasting results would indicate that indigenous cells (at least a fraction of them) have surface properties which are more similar to humus (possibly coated by humic compounds) compared to the recently introduced Pseudomonas cells.

High cation concentration, and in particular di- and trivalent cations, is disastrous for cell extraction because it may cause flocculation of negatively charged colloids, and hence coprecipitation of bacteria and soil colloids (which are both negatively charged). We have stressed earlier that for most soils a sufficiently low di- and trivalent ion concentration can be achieved by dispersion in distilled water (Lindahl and Bakken 1995), rather than using ion exchangers proposed by MacDonald (1986). Thus dilution (less soil per ml) is an easier solution to the flocculation than adding ion exchangers.

Low concentrations of di- and trivalent cations help to avoid flocculation but do not help much in dislodging bacteria that are strongly attached to soil particles (Bakken and Lindahl 1995).

The pH in the soil slurry during dispersion and separation has an invariable effect on cell extraction; within the pH range 5–8 the cell yield increases substantially with increasing pH. So does the "yield" of humus, however, and for the same reason; the density of negative charges on humus and organisms increases as pH is raised, resulting in stronger electrostatic repulsion within humus colloids, hence expanding and dissolving humus, desorbing cells and hindering flocculation. Our experience (unpubl. res.) is that for soils which give a low cell yield (much less than 10–15%), great improvement can be achieved by a moderate increase in pH (to 7 or somewhat higher). One way to achieve a high pH (most soils are acidic) is to disperse the soil in weak NaOH (the necessary concentration can be determined beforehand by titration of a soil suspension). Alternatively, the soil may be dispersed in 20 mM sodium phosphate solution (Na_2HPO_4, pH 7.5–8.5).

Extracellular structures and polymers participate in establishing irreversible attachment of bacteria to surfaces, and the use of enzymes could help in releasing the cells (Bøckelmann et al. 2003). The problem is that enzymes are specific and that the polymers involved in bacterial attachment in soil are probably very diverse; it is a challenge to find the right mixture! Another problem is that enzymes may have an adverse effect on bacteria. Bøckelmann et al. (2003) designed a cocktail of lipase, α- and β-glycosidase to help in dispersing soil bacteria. The effect on dispersion was substantial when used in a protocol with only mild physical dispersion (rotary shaking), but the enzymes efficiently killed three (one G+ and two G−) out of ten tested bacterial cultures. A tentative conclusion is that the use of enzymes may improve cell yields but much work remains before they can be used, either for non-selective extraction or for deliberate selective extraction of certain cells. The latter appears to be the most plausible option.

3.5.2 Separation

Bacteria can be separated from soil particles on the basis of differences in sedimentation rates, albeit with an ill-defined cut off and moderate success regarding purity of the bacterial suspension (Lindahl and Bakken 1995). The method of Fægri et al. (1977) was originally designed for separating bacteria from fungi, the sedimentation rate chosen to ensure that all fungal spores would sediment together with the majority of the soil particles (Bakken 1985). For this reason, fungal cells are unlikely to be separable

from soil on the basis of sedimentation rates. Quantitative separation of fungi from soil is probably extremely difficult, if possible at all (Bingle and Paul 1986). Sieving has been used for decades to separate fungal spores from hyphae in soil, the original purpose being differential isolation (culturing) of fungi derived from spores versus hyphae (Watson 1960; Gams and Domsch 1967; Fritze and Baath 1993; Mori et al. 2000). It would be equally feasible with nucleic acid methodology to differentiate between fungal DNA (or RNA) present in spores and hyphae.

The buoyant density of cells is different from soil particles, and this has been exploited for separation of cells from soil. All structures that contain water have a characteristic density that depends on their ability to exclude the solutes (or colloids) in the surrounding medium. Thus, all intact cells have a moderately low density and are hence separable from soil particles, provided that the membrane is able to exclude the solutes of the medium and that its osmolarity is low enough to avoid plasmolysis (dehydration of the cytoplasm). Neither CsCl nor sucrose solutions fulfill this last criterion, in contrast to suspensions of colloidal silica (Bakken 1985) and solutions of the non-ionic and non-toxic large molecular weight benzoic acid derivative Nycodenz (Bakken and Lindahl 1995). Low viscosity is another characteristic of the two density-gradient media (Percoll and Nycodenz) which makes them very useful in separating cells from soil. The separation of intact cells from soil particles by high-speed centrifugation (10,000 g for 0.5–2 h) on high-density cushions (Bakken and Lindahl 1995) is both a simple and robust method to separate “clean” cells from soil colloids and “dirty” cells (coated by or attached to soil materials). For unknown reasons, fungal spores, and in particular fungal hyphae, appear to be virtually absent from the fractions. Protozoa have been observed to float on top of the Nycodenz cushion (flagellates and amoebae, unpubl. res.), but not checked in any quantitative way. The possibility to “float” off protozoa and fungal structures after milder dispersions than those used till now would be well worth a try!

A one-step dispersion and separation of indigenous bacterial populations with this method has proved to give a reasonably representative fraction (10–20%) of the total bacterial flora, as tested by quantifying broad phylogenetic groups with hybridisation against extracted DNA (Courtois et al. 2001), by physiological profiling with respect to heterotrophic metabolism (Mayr et al. 1999), culturability and the metabolic activity per cell (Lindahl et al. 1996). Flow cytometric analysis of indigenous soil bacteria has also become possible (Christensen et al. 1993, 1995). Further, one-step dispersion and separation was shown to extract a near 100% of cultured cells added to soil suspensions (Lindahl and Bakken 1995), which makes it an ideal method to extract reporter bacteria for flow cytometric studies (Unge et al. 1999; Burmølle et al. 2003).

As stressed before, results of cell extraction should be interpreted with caution. The extracted cells represent a fraction of the whole community (except perhaps for organisms recently added to the soil), and it cannot be taken for granted that they represent the total. However, this bias can be exploited as a source of new knowledge, rather than just another methodological problem. Attempts to extract ammonia- and methane-oxidising bacteria demonstrated that these numerically minor (but ecologically important) groups are strongly attached to soil particles (Priemé et al. 1996; Aakra et al. 2000), but less so for newly grown cells (Aakra et al. 2000). Targeted extraction of methane-oxidising bacteria was utilised to demonstrate a much lower pH optimum for indigenous methanotrophic bacteria than that for any known cultured methanotroph (Amaral et al. 1998). Biased extraction of bacteria according to their position inside or on the surface of aggregates was used to demonstrate that cells inside aggregates are protected against spikes of mercury (Ranjard et al. 2000). Similarly, we have recently compared strongly and loosely attached cells as to their trace metal tolerance (Almaas, Mulder and Bakken, manuscript in preparation). The loosely attached bacteria developed a normal pollution-induced tolerance (PICT; Blanck 2002; Diaz-Ravina and Baath 2002) to Zn, whereas the strongly attached showed no response to Zn pollution. The results shed light on the importance of positioning in the soil matrix for growth versus survival of bacteria. The last few examples bring us back to our introductory citation of Leonard Cohen: The bias of the method (the crack) can be *used* rather than avoided, so as to increase our understanding of the biology of soils (the light comes in).

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4 Role of Stabilised Enzymes in Microbial Ecology and Enzyme Extraction from Soil with Potential Applications in Soil Proteomics

Paolo Nannipieri

4.1 Introduction

The same enzyme can have different locations in the soil matrix being cytoplasmic, periplasmic (in Gram-negative bacteria) or attached (ectoenzymes) to the outer surface of active cells, intracellular in dead cells or cell debris, intracellular in resting cells, such as bacterial spores or protozoan cysts, or released as extracellular enzymes, which can be free in the soil solution, adsorbed on a substrate or on soil colloids (Burns 1982; Nannipieri 1994; Chap. 12). Some of these enzyme activities are not quantitatively important; e.g. "free" extracellular enzymes are supposed to be short-lived unless they are protected from proteolysis upon adsorption by soil minerals or entrapment by humic colloids (Burns 1982; Nannipieri et al. 2002). It is well established that enzyme activities of resting cells, such as bacterial spores, are very low and probably undetectable in soil enzyme assays (Atlas and Bartha 1981). A better interpretation of measurements of enzyme activities should be possible by distinguishing the extracellular activity due to enzymes adsorbed by soil minerals or entrapped by humic colloids from the activity of enzymes associated with active microbial cells (the cytoplasmic and periplasmic enzymes and the ectoenzymes; Nannipieri et al. 2002).

Of the different locations that an enzyme can have in the soil matrix, those associated with soil colloids have been extensively studied because they are markedly resistant to thermal denaturation and generally to proteolysis (Nannipieri et al. 2002). Thus, they can be active even under conditions limiting microbial activity and are not regulated by factors repressing or derepressing their synthesis. For this reason they are also called "stabilised" enzyme activities in soil. Research aimed at understanding the state of these enzymes in soil has been carried by two different approaches. The first concerns the study of properties of enzyme complexes prepared in the laboratory. For example, various enzymes have been adsorbed on pure clay minerals made homoionic to different cations (Stotzky 1986; Nannipieri et

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al. 1996; Chap. 7). Model humus–enzyme complexes have been prepared by entrapping enzymes during oxidative coupling or condensation reactions of phenolic compounds (Burns 1986; Nannipieri et al. 1996). The second approach concerns the extraction of enzyme complexes from soil with successive studies of the properties of the extracted complex. Only organic enzyme complexes or free enzymes have been extracted from soil (Nannipieri et al. 1996). Clay–enzyme complexes have not been extracted from soil and, as discussed below, their presence has not been detected by electron microscopy; however, there is indirect evidence that they can exist in soil.

The present knowledge on the extraction of enzymes from soil can also be used in studies about soil proteomics, which involves protein extraction from soil. There is the need to combine studies on soil functionality with genome characterisation, i.e. to relate the presence of genes with their expression, and this can be carried out not only by characterising DNA and mRNA extracted from soil, but also by monitoring protein syntheses and specific soil functions (Nannipieri et al. 2003; Smalla 2004). It is well established that the statement "one gene one protein" is no longer valid because several events can occur between gene expression and protein functions (Graves and Haystead 2002; Fig. 4.1). The mRNA molecule can undergo post-transcriptional control due to alternative splicing, polyadenylation and mRNA editing (Sarkar 1977; Newman 1998). Thus different protein isoforms can be generated from a single gene at this stage. Some stable mRNA-like transcripts (spliced and polyadenylate) are non-coding and function as RNA riboregulators (Erdmann et al. 1999). In addition, mRNA can be subjected to regulation and then the formed protein can be subjected to post-translational modifications and regulated by proteolysis (Kirschner 1999) and compartmentalisation (Colledge and Scott 1999). According to Krishna and Wold (1993), there are 200 different types of post-translational modifications.

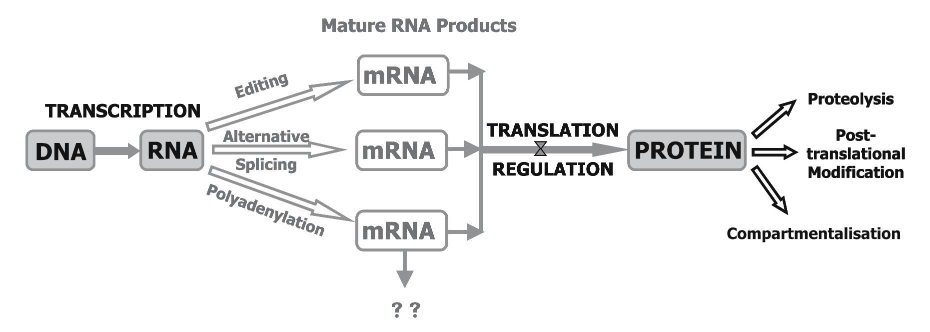


Fig. 4.1. Several processes occurring between gene expression and function of physiologically active proteins (partially modified from Graves and Haystead 2002)

The aims of this review are to discuss: (1) the indirect evidence that supports the presence of active enzymes adsorbed by clay minerals or entrapped by humic molecules in soil; (2) the various methods used to extract "stabilised" enzymes from soil and the characterisation of the extracted complexes; (3) the role of stabilised enzymes in microbial ecology; (4) the application of proteomics to soil with the manifold problems due to the complexity of the system; and (5) the contribution of the existing knowledge on the extraction of enzymes from soil to set up efficient and conceptually correct methods of protein extractions from soil. As for the application of any innovative technique and approach to soil, a successful application of proteomics to soil requires careful consideration of the complexity of the soil system. Of course, since soil proteomics is still in its infancy, the discussion on soil proteomics will be mostly speculative and it will partly overlap with what is discussed in Chap. 5.

The extraction of enzymes from soil has been carried out with the aim of understanding the mechanisms responsible for the formation of organo-mineral complexes in soil and the protection of enzymes by soil colloids against proteolysis. Thus, attention has been focused on extracellular hydrolases which could be active in the extracellular environment and whose activity could be determined in soil. Hydrolase activities were also selected for their importance in nutrient cycling. Thus the most studied enzymes were urease, phosphatases, proteases, β-glucosidases, etc. In contrast, the attention of proteomics might be focused on specific enzymes which respond to changes in environmental factors and soil pollution or can play important roles in biotic processes. Of course the two approaches are not exhaustive and other aspects, such as the origin of enzymes, still require further research.

4.2 Evidence for the Presence of Stabilised Enzymes in Soil

The most important piece of evidence of the presence of stabilised enzymes in soil derives from studies on the nitrogen (N) distribution in soil carried out in the 1960s and 1970s and based on the use of acid hydrolysis of soil (Bremner 1965; see also Chap. 2). Amino acids are the main identifiable organic N compounds in soil hydrolysates, where they can range from 30 to 45% of total soil N (Stevenson 1986; Schulten and Schnitzer 1998). However, net N mineralisation, i.e. the amount of N made available to plants, accounts for 2% of the total N, and it corresponds to 100–200 kg N per hectare, a normal rate of N fertilisation (Stevenson 1986). An intriguing question is how this organic N is slowly mineralised. A better understanding of the state of proteins and protein–colloid complexes in

soil can clarify mechanisms of the N mineralisation. By considering that the percentage of total N in microbial biomass averages to 4% (Jenkinson 1988) and that acid hydrolysis breaks peptide bonds, it is reasonable to hypothesise that most of the amino acid N in soil hydrolysates derives from extracellular protein N stabilised in soil by soil colloids. The percentage of total N present as amino acid N (85%) determined by ^{15}N NMR was even greater than that found after acid hydrolysis of soil (Knicker et al. 1993; Schulten and Schnitzer 1998). However, direct analysis by ^{15}N NMR spectrometry is difficult because natural ^{15}N abundance is very low (0.366%; see Chap. 2).

The visualisation by scanning electron microscopy of soil sections prepared by ultracytochemical tests has allowed the detection of enzymes such as acid phosphatase, succinic dehydrogenase, peroxidase, and catalase in root and microbial cells and acid phosphatase in fragments of microbial membranes as small as 7×20 nm (Foster and Martin 1981; Foster 1985; Ladd et al. 1996). Unfortunately, the presence of electron-dense soil components, such as minerals, or soil components, such as humic molecules, which aspecifically react with the counterstainer OsO_4, caused problems for the detection of enzymes adsorbed by clay minerals or englobated by humic complexes. These problems were partially overcome by using electron probe microanalysis and proper controls, where the enzyme was inhibited or the substrate not added (Ladd et al. 1996). By using this approach no phosphatase activity was found on quartz particles while enzyme activity associated to microbial cells was detectable, and there was also little evidence for the presence of phosphatase activity associated to clays (Ladd et al. 1993).

However, it is well established that the contents of organic matter and microbial biomass depend on the clay content of soil (Ladd et al. 1996). Already Jenny in 1941 concluded that the clays retard the decomposition of organic matter by comparing soils differing in clay content but with similar climate, vegetation and management regimes. The addition of ^{14}C-labelled simple (such as glucose) or complex (vegetable remains) substrates to soils with varying clay content has led to the understanding that the clays do not affect the prime degradation of the substrate but the successive stabilisation of the microbial degradation products and the synthesised microbial biomass (Ladd et al. 1996). Differences in the dynamics of C and N turnover were assessed by studies based on fractionation procedures of soils treated with ^{14}C- and ^{15}N-labelled compounds. Extracellular N organic compounds produced by microorganisms were preferentially associated with clay particles during early periods of microbial degradation of ^{14}C- and ^{15}N-labelled wheat straw or ^{14}C-labelled glucose added with ^{15}N-NH_4^+ (Ladd et al. 1996). As shown in Chap. 7, the bibliography on the properties of protein–clay complexes prepared in the laboratory is extensive.

4.3
Extraction of Enzymes from Soil

A cathepsin-like activity was measured in a soil extract in 1954 (Antoniani et al. 1954). However, according to Skujins (1967), Fermi and Sabramanian were the first to measure enzyme activities (protease and aminase activities, respectively) in a soil extract. Urease was isolated from a surface forest soil with phosphate buffer at pH 6 and purified in the United States by Briggs and Segal (1963). In Australia, Ladd (1972) determined protease and peptidase activities in several pasture and wheat soil extracts by using 0.1 M Tris-borate buffer at pH 8.1 as an extractant. Then, in the 1970s and 1980s, lyase, oxidoreductase and hydrolase activities were monitored in soil extracts (Nannipieri et al. 1996). Several procedures for the extraction of enzymes from soil have been proposed and they are generally based on the use of salt solutions as extractants. Phosphate, acetate, citrate, tris(hydroxymethyl)aminomethane (tris), tris-borate, borate, etc., have been used to extract enzymes from soil, as reviewed by Shcherbakova et al. (1981), Tabatabai and Fu (1992) and Nannipieri et al. (1996). It was realised that it is not acceptable to purify enzymes extracted from soil because the extracted enzymes can be a mixture of enzymes from different sources (Nannipieri et al. 1996). Indeed, enzymes can originate from a multitude of sources from the variety of microbial species inhabiting soil, and from plants and fungi (Torsvik et al. 1996; Nannipieri et al. 2003). It was suggested that enzyme extraction from soil should give high yields of extracted enzymes to allow the characterisation of the properties of the extracted enzyme complexes (Nannipieri et al. 1974, 1980, 1996). In addition, an efficient extraction procedure for immobilised enzymes in soil should avoid the lysis of microbial cells (and thus the consequent release of intracellular enzymes), the formation of artefacts during soil extraction, such as the adsorption or entrapment of the extracted enzymes within the solubilised complexes, and interaction between the extractant and the inorganic components to give enzyme-like activity (Nannipieri et al. 1996). For example, the citrate can interact with manganese to form complexes showing laccase-like activity as a result of soil extraction (Leonowicz and Bollag 1987). Nannipieri et al. (1974) used sodium pyrophosphate at neutrality to extract ureases from a humic podzol without breaking microbial cells, by considering that this solution had been used to extract organic matter under mild conditions (Bremner and Lee 1949). Sodium pyrophosphate was also used to extract urease from coarse textured solodic, reddish-chocolate clay loam, grey-brown podzolic, red-yellow podzolic and alpine humus soils (Lloyd 1975). The same extractant was used to extract β-glucosidase and catalase from an Umbric Dystrochrept soil (Perez Mateos et al. 1988; Busto and Perez Mateos 1995), and phosphatase, casein- and

benzoylargininamide-hydrolysing proteases from Mollisol, Histosol and Alfisol soils (Nannipieri et al. 1980).

Sodium hydroxide, the common solution used to extract organic matter from soil at alkaline pH values (12–13), was used to extract malathion esterase from a clay-loam soil; the enzyme was then purified by a procedure involving addition of salts ($MnCl_2$), with precipitation by $(NH_4)_2SO_4$, dialysis and ion chromatography. It was shown that the extracted enzyme was a glycoprotein (Getzin and Rosefield 1971; Satyanarayana and Getzin 1973). However, alkaline conditions should be avoided in studies on enzyme–soil colloid complexes because enzymes are denatured and lysis of microbial cells can occur.

Extensive characterisation of extracted enzymes from soil has been mainly carried out by the groups of Mayaudon in Belgium and Nannipieri and Ceccanti in Italy with the aims of studying properties and state of the organic-enzyme complexes and the processes responsible for their formation (Nannipieri et al. 1996). The two groups used different extraction procedures and thus probably studied different enzyme complexes.

Laccases, polyphenol oxidases, phosphatases, phosphodiesterases, arylsulphatases, cellulases, xylanases, β-glucosidases, invertases and proteases, extracted by shaking a pasture soil with phosphate-EDTA at pH 7–8 for 1 h (Mayaudon et al. 1973; Batistic et al. 1980), were supposed to be fungal glycoenzymes protected against proteolysis by their entrapment in lipopolysaccharides synthesised by Gram-negative bacteria (Mayaudon 1986). It was also hypothesised that these multienzymatic lipopolysaccharide complexes were linked through Ca bridges to the humic matrix and their extraction by phosphate occurred because EDTA chelated these Ca ions (Fig. 4.2). According to Mayaudon (1986), the interactions between the fungal enzymes and the bacterial lipopolysaccharides occur after partial hydrolysis of the latter once released into soil environment.

The enzymatic preparations extracted by pyrophosphate were rich in humic matter; these humic-enzyme complexes were fractionated by gel chromatography only after exhaustive ultrafiltration of the soil extract with sodium pyrophosphate at pH 7.1 (Ceccanti et al. 1978). The compounds with molecular weight lower than 10,000 were discarded and the retained material was separated by ultrafiltration into two fractions of molecular weight higher (A_I) and lower (A_{II}) than 100,000. Gel chromatography of these two fractions (A_I and A_{II}) gave three and two urease active fractions differing in molecular weight, respectively (Ceccanti et al. 1978). The gel chromatography of the highest molecular weight fraction (A_I) with sodium pyrophosphate as eluent gave three peaks of phosphatase activities and one peak of each protease (casein and benzoylargininamide-hydrolysing proteases) activity, whereas the gel chromatography of the lowest molecular

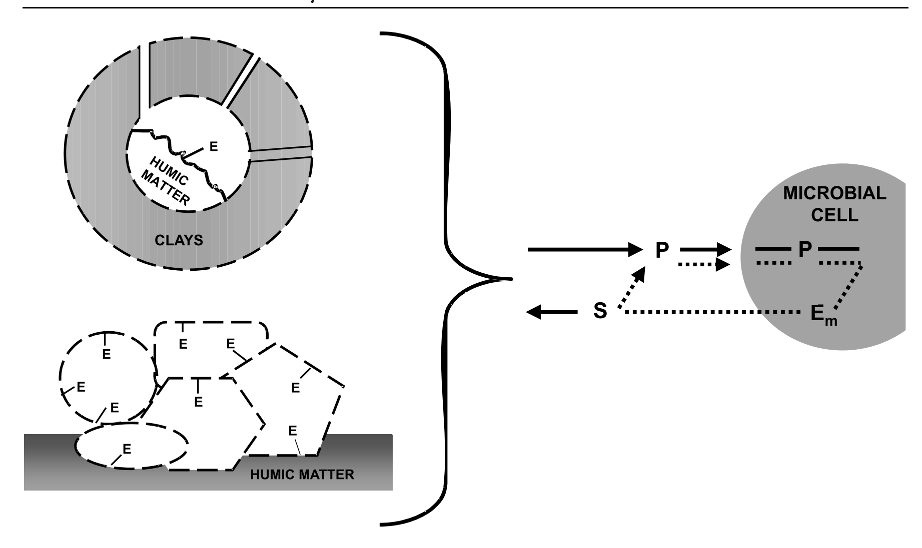


Fig. 4.2. Possible role of stabilised enzymes (model by Burns above; model by Mayaudon below) in microbial ecology (modified by Burns 1982). *P* Product; *S* substrate; E_m induced microbial enzyme; *E* enzymes

fraction (A_{II}) using water as eluent gave one peak of each enzyme activity (Nannipieri et al. 1985).

Characterisation of soil extracts by pyrolysis-gas chromatography (Py-GC) showed that 0.1 M sodium pyrophosphate at pH 7.1 extracted condensed humic substances, glycoproteins, intact or partially decomposed carbohydrates (Bonmati et al. 1988). Soil extracts were active against three different protease substrates: *N*-benzoyl-L-argininamide, specific for trypsin, *N*-benzyloxycarbonyl-L-phenylalanyl L-leucine (ZPL), specific for carboxypeptidases, and casein, essentially non-specific.

Characterisation of A_I and A_{II} fractions by elemental analysis, Py-GC and isoelectric focusing (IEF) showed that both fractions had a C/N ratio higher than a pure protein and properties similar to humic and fulvic acids of the same soil (Ceccanti et al. 1986). In addition, the A_I fraction was richer in carbohydrates and in highly condensated humus than the A_{II} fraction. Total activity of these hydrolases was generally increased after ultrafiltration or gel chromatography, probably as the result of separation of inhibitory humic constituents and enzymes (Nannipieri et al. 1985). Phosphatase and urease active fractions with higher molecular weight were more resistant to thermal denaturation and proteolysis than the enzymatically active fractions with lower molecular weight (Nannipieri et al. 1978, 1982, 1988). It was hypothesised that these data validated the hypothesis by Burns et al. (1972a) who proposed that hydrolases (ureases) were entrapped within or-

ganic or organo-mineral complexes and surrounded by a humic network with pores large enough to allow the passage of substrates and products of the hydrolytic reaction, but not the passage of high molecular weight compounds such as proteases (Fig. 4.2; Nannipieri et al. 1996).

Usually, studies on enzyme extraction from soil and characterisation of enzyme complexes have mainly focused on hydrolases (urease, phosphatase, β-glucosidase, etc.) for their important role in nutrient cycling and for the extensive bibliography on properties of the respective model complexes such as those with clays (see Chap. 7). It is important to underline that the present knowledge in the field derives above all from studies carried out in the 1970s and 1980s. In addition, it is well established that artefacts can occur during the extraction of enzyme complexes from soil.

4.4 The Role of Stabilised Enzymes in Soil Microbial Ecology

According to Burns (1982) enzymes entrapped by humic colloids are essential for the successful competition of microbial cells in the hostile soil extracellular environment. Particularly the stabilised enzymes can catalyse the transformation of high molecular substrates into products that can be taken up by near microbial cells and can act as inducers or chemoattractants. In the first case the products induce the microbial cell to synthesise and release the specific extracellular enzyme into the extracellular soil environment; in the second case, the microbial cells will move towards the substrate.

Both Mayaudon's and Burns' models (Fig. 4.2) involve the entrapment of the stabilised enzymes by organic polymers or organo-mineral complexes, with the surrounding networks having pores large enough to permit the passage of low molecular weight substrates and products but not the passage of the high molecular weight proteases, which should catalyse the proteolytic hydrolysis of the stabilised enzyme. This view has been criticised by Ladd and Butler (1975) for extracellular enzymes acting on high molecular weight substrates because such type of protection would also not permit the detection of the stabilised enzymes.

According to Burns (1982) it is difficult to prove the validity of any theory interpreting processes at the microenvironment scale in soil because we are not able to set up model experiments simulating such a reality. An interesting technique to study spatial interactions between microorganisms, enzymes and their substrates was set up by Hope and Burns (1985). Petri dishes were prepared with carboxylmethylcellulose (CMC) agar; after solidification, a ring of agar was removed and replaced by a barrier ring prepared with soil fractions or soil. Cellulase was pipetted inside the bar-

rier ring and the extent of diffusion of the enzyme outside the barrier was measured by precipitating any unhydrolysed CMC. Clay minerals such as bentonite, with a high cation-exchange capacity and a high unit surface area, limited the diffusion of cellulase, whereas kaolinite, with properties opposite to bentonite, had no effect on the diffusion of the enzyme.

4.5 Proteomics

Proteomics, a term coined in 1995, aims to obtain a global and integrated view of the biology of cells by studying all the proteins of a cell rather than each one individually (Wasinger et al. 1995; Wilkins et al. 1995). As stated in Sect. 1, there are many more proteins than genes in cells because of post-transcriptional modifications. This implies that the gene characterisation is not sufficient to assess the types and functions of proteins in the organism.

Complete draft sequences are now available for the genomes of many eubacteria, several archaebacteria, several unicellular eukaryotes, several plants and animals. As these sequences have accumulated it has become increasingly apparent that new methods are needed to exploit the information that they contain. Three types of proteomics have been applied in biology (Graves and Haystead 2002). *Protein expression proteomics* concerns the quantitative study of protein expression between samples that differ by some variables. This approach has allowed the determination of specific proteins in signal transduction or in disease processes. *Structural proteomics* aims to identify all proteins of a protein complex (e.g. in subcellular organelles) assessing their location and characterising all protein–protein interactions. *Functional proteomics*, a broad term for many specifically directed proteomic approaches, allows the study and characterisation of a selected group of proteins and can provide important information about protein signalling, disease mechanisms or protein–drug interactions. Thus, the proteomics approach can concern either the entire proteome or subproteomes.

In any approach, the analytical method should resolve the protein mixture into its individual components so that each protein can be identified and characterised. The predominant method used for the separation of proteins is two-dimensional (2-DE) polyacrylamide gel electrophoresis where proteins are separated by two distinct properties: the net charge in the first dimension and the molecular mass in the second dimension (Graves and Haystead 2002). The immobilisation of a pH gradient on the gel has increased the resolution and reproducibility of the technique (Bjellqvist et al. 1993; Gorg et al. 2000). In particular, 2-DE has the ability to resolve proteins that have been subjected to post-translational modifications, because these

protein modifications confer a change in protein mass and charge (Graves and Haystead 2002). The main application of the technique is the comparison between two samples so as to determine qualitative and quantitative differences concerning the protein expression. Indeed, the appearance or disappearance of spots can provide information about differential protein expression, while the intensity of the spots can give quantitative information about protein expression levels (Graves and Haystead 2002). A recent advance in 2-DE is represented by difference gel electrophoresis (DIGE) where two proteins fluorescently tagged with two different dyes are run on the same 2-D gel (Unlu et al. 1997). After the run, fluorescence imaging of the gel is used to create two images which are superimposed to identify pattern differences. Thus this approach avoids comparison of several 2-D gels.

The drawbacks of 2-DE are: (1) the technique is labour-intensive and time-consuming; and (2) the complete resolution of all proteins does not occur on a single 2-D gel because the protein mixture is too complex to be completely resolved (Graves and Haystead 2002). Large or hydrophobic proteins will not enter the gel during the first dimension, and the pH range of the gel does not permit proteins with isoelectric points (Ip) lower than pH 3 and higher than pH 10 (Gorg et al. 2000) to be resolved. In addition, the gels only show abundant proteins and not low-copy proteins.

An alternative to 2-DE is to digest a protein mixture to peptides by trypsin and then to purify the peptides before analysis by mass spectrometry (MS). The disadvantage is the cost of the instrumentation, the time employed in the analysis and the computing power to deconvolute the data obtained (Graves and Haystead 2002). This approach presents the advantage that it is able to analyse a greater number of proteins than the 2-DE method. In addition, the mass spectrum of the unknown protein can be compared with theoretical mass spectra produced by computer-generated cleavage of proteins in the database (Graves and Haystead 2002).

Another promising alternative to 2-DE is the use of an isotope-coded affinity tag (ICAT) which allows quantitative profiling between different samples without the use of electrophoresis (Gigy et al. 1999). Protein samples are treated with two chemically identical reagents that differ only in the mass as a result of the isotope composition. The ICAT reagent consists of a biotin affinity group, a linker region that can incorporate heavy (deuterium) or light (hydrogen) atoms, and a thiol-reactive end group for linkage to cysteine residues of proteins. This reagent permits the quantification of the expression level of proteins (Graves and Haystead 2002). For example, two sets of populations of cells in a different state can be differentiated because they are labelled with either a light or heavy form of the ICAT reagent, and the difference in peak heights between heavy and light peptide ions directly correlates with the difference in protein abun-

dance in the cells. In addition, the protein can be identified after hydrolysis by trypsin, purification of labelled peptides by avidin chromatography by virtue of the biotin tag, and analysis by MS.

Another promising technique is microarray systems involving miniature chips and MS, which allow the analysis of a great number of proteins (see Chap. 5).

4.6 Soil Proteomics

It is now recognised that also in soil microbiology there is a need to go beyond DNA analysis for a better understanding of soil functionality (Nannipieri et al. 2003). By characterising both DNA and RNA one might be able to determine the actual species effectively involved in the processes being measured. Different protocols involving the simultaneous extraction of RNA and DNA from soil have recently been published (Duarte et al. 1998; Griffiths et al. 2000; Hurt et al. 2001; Weinbauer et al. 2002; see also Chap. 3). Further research is required for the efficient removal of humic substances and residual DNA without partial degradation of the RNA so as to reliably use RNA for microbial community analysis (Smalla 2004). However, as mentioned above, there is a need to combine the proteomics approach with nucleic acid characterisation to better understand soil functions.

Microbial proteomics has mainly concerned axenic cultures whereas environmental applications are still in their infancy (Graves and Haystead 2002). A correct application of proteomics to soil should consider the complexity of the system; only 4% of total N is present in microbial biomass whereas 30–45% of the soil N is present as extracellular proteins (Stevenson 1986). Thus, the study of gene expression by soil microbiota is made complex by this high background of protein N stabilised by soil colloids which is not directly related to microbial activity.

By considering the distribution of organic N in soil the author would suggest an arbitrary classification of soil proteomics into *functional proteomics* and *structural proteomics* (Fig. 4.3). The former should reveal differences in protein expression by soil microflora in the presence of pollutants and organisms (fauna, plants and other microorganisms) interacting with the target microbial species, changes in agricultural or forest management and changes in environmental conditions. It should evidence any protein responsible for homologous cell-to-cell interactions, such as quorum sensing, and proteins involved in the heterologous cell-to-cell interactions, such as symbiosis and competition. It should reflect microbial activity and it should be combined with DNA and mRNA analyses to link the expression of specific gene sequences to the respective protein function. One possible

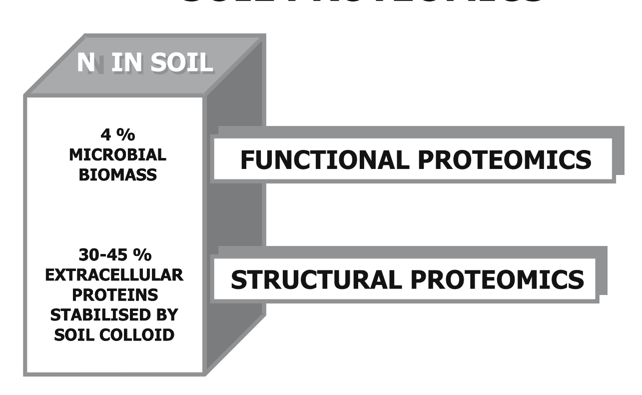


Fig. 4.3. Possible proteomics approaches in soil

problem may be the presence of different proteins (from diverse microbial sources) with the same function. In contrast, *structural proteomics* should characterise proteins adsorbed and stabilised by soil colloids so as to understand mechanisms responsible for such stabilisation in situ. Indeed, most of our knowledge on this subject derives from laboratory experiments involving interactions of a single pure protein with a single colloid (clay mineral or humic fraction). This is not the situation *in situ* because, for example, "dirty" proteins (due to the presence of other cellular components) interact with "dirty" clays (clays covered by hydroxides, oxides and/or humic molecules). So far only the adsorption of pure proteins versus "dirty" clays has been compared (Stotzky 1986; Fusi et al. 1989; Nannipieri et al. 1996).

Murase et al. (2003) found that the clearest electrophoretic patterns in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) were obtained when extracellular proteins were extracted from an Entisol with phosphate buffer at pH 6.0. Protein bands ranged from 35 to 68 kDa and a homology search in the Protein Information Resource International Database, through the GenomeNet (http://fasta.genome.ad.jp/), allowed the identification of a homologue of thermostable cellulase produced by the thermophilic fungi *Humicola insolens* and *H. grisea* (Murase et al. 2003). By considering the extracting solution used, Murase et al. (2003) probably investigated free extracellular and intracellular proteins released by cell lysis during the extraction. Indeed, phosphate at pH 6.0 is a poor extractant of organic matter from soil and the extraction of stabilised proteins requires more efficient extractants and higher pH values, as discussed above. By increasing the pH of the phosphate extractant, Murase et al. (2003) increased the amount of extracted organic matter from soil resulting in SDS-PAGE patterns with fuzzy protein bands and tailing.

Another successful application of proteomics to soil was shown by Singleton et al. (2003) who showed that Cd pollution decreased the total amount of protein extracted by 35% and increased the production of small molecular weight proteins (< 21 kDa), probably indicative of a microbial response to metal exposure (see Chap. 5). Proteins were extracted after four repeated freeze/thaw cycles to kill and lyse cells in the presence of a protease inhibitor cocktail.

An autoclaving method with citrate buffer at pH 8.0 was used to extract glomalin from soil (Wright and Upadhyaya 1996, 1998; Rilling et al. 2002). Properties and the supposed role of glomalin in soil are discussed in Chap. 5.

Proteins of dissolved organic matter (DOM) have been characterised by MS-based proteomics (Schulze et al. 2005). Their phylogenetic origin and metabolic functions were assessed by using an extensive database. The approach involves removal of humic acids and small molecules from soil solution by gel filtration, proteins separation by SDS-PAGE, trypsin digestion of protein gel slices, extraction of tryptic peptides from gel slices, with their desalting and separation by nanoflow liquid chromatography prior to analysis by MS. About 50% of the proteins of forest soil DOM originated from bacteria and the number of identified proteins decreased with depth and with the amount of DOM. A protease inhibitor cocktail was not used during DOM sampling at different soil depths and active proteases might have affected the protein composition of the sample.

The MS-based proteomics was applied to characterise soil enzymes involved in C reactions (Schulze et al. 2005). After air-drying and sieving (< 2 mm) of soil, discrete organic particles were eliminated by flotation, and settled soil material was washed with deionised water and then air-dried. Proteins were separated from inorganic material by 10% HF, and, after neutralisation, they were separated from the other organic molecules by gel filtration and SDS-PAGE before being analysed as reported above.

The use of ultrasonic treatment proposed by Ogunseitan (see Chap. 5) can increase the extraction efficiency of the phosphate extractant but it also favours the solubilisation of stabilised proteins because it breaks soil aggregates. Thus, a mixture of microbial and stabilised proteins is obtained in the soil extractant. The same drawback can be present in the procedure by Schulze et al. (2005) to extract enzymes from soil by using HF solution which is a strong acid extractant. As discussed above, extracts with only microbial proteins should be obtained if the aim is to study protein expression (functional proteomics). Given the large background of stabilised proteins in soil, a possible approach can be to separate microbial cells from soil particles followed by the extraction of proteins from the microbial cells. Possible drawbacks of the cell extraction method might be the low extraction yields and possible changes in the composition of

protein mixture during the extraction procedure. An advantage might be the possibility to use techniques set up with axenic cultures, such as the ICAT technique. It might be possible to label proteins, DNA and mRNA molecules, with the possibility to distinguish and separate labelled from unlabelled molecules. Labelling of nucleic acids with stable isotopes is discussed in Chap. 10. Methods based on freeze/thaw or autoclaving, used by Singleton et al. (2003) and Wright and Upadhyaya (1996), may mainly extract intracellular or ectoproteins since these techniques cause cell lysis, and the extraction of proteins associated with soil colloids is low by the buffer solutions used (Nannipieri et al. 1996). However, the addition of a protease inhibitor cocktail is needed because proteases are active during cell lysis in soil, as shown by Renella et al. (2002), and they can hydrolyse the target protein. In addition, contaminants co-extracted with proteins from soil can be present and they should be eliminated by purification procedures before protein analysis. This procedure might cause the loss of specific proteins or non-proteic components essential for the function of the target protein. Anyway, each extraction method should also show a recovery yield of known proteins added to soil and extracted by the method used so as to determine the extraction efficiency of the employed procedure. Indeed, the quantitative recovery of the target protein is essential to obtain consistent data.

So far there are no studies on structural proteomics with precise identification of proteins really involved in the adsorption or binding with soil colloids. Humic-enzyme complexes have been extracted from soil, partially purified and characterised as reported above but their organism origin, and thus the type of enzyme, has not been determined. Enzyme-humic complexes, extracted from soil and partially purified, have been fractionated by IEF. There are also a few reports of soil structural proteomics. Cacco and Maggioni (1976) extracted acetylnaphthyl esterase from an A_1 horizon of an Alpine podzol using the procedure of Burns et al. (1972b). Briefly, they carried out a first extraction with 0.25 M phosphate buffer (pH 7.0), 2 M urea, 4 M NaCl, 0.013 M ethylenediaminetetraacetate and 0.06 M mercaptoethanol, at 4 °C for 4–5 h; a second extraction of the soil sediment with 0.25 M phosphate (pH 7) for 3 h; a third and fourth extraction with 0.05 M phosphate for 3 and 4 h, respectively. The pooled soil extract was dialysed against 5 mM mercaptoethanol and concentrated by dialysing it against polyethylene glycol before IEF. Both bands at Ip values of 5.7 and 6.5 showed enzyme activity and contained humic molecules, whereas the bands at Ip 4.7 and 5.7 only showed enzyme activity (Cacco and Maggioni 1976). No enzyme activity was observed below Ip 4.7.

IEF of humic matter and proteases (benzoyl-L-arginimide-hydrolysing proteases) of soil extracts (obtained with sodium pyrophosphate at pH 7.1) and derivative fractions (A_I and A_{II}, see above) obtained by ultrafiltration

was carried out by Ceccanti et al. (1989) on 5% (w/v) polyacrylamide gel tubes (0.5 × 8 cm) using carrier ampholytes at pH 4–6 at a final concentration of 2%. Densitometric scanning of the focused organic matter was carried out at 460 nm with a densitometer, and gel-trapped humic bands were analysed for the protease activity. The largest proportion of protease activity in the extract was located in a broad organic-matter peak at Ip 4.44. About 50% of the enzyme activity was found in the remaining humic bands, whose Ip values ranged from 4.93 to 4.06. However, the highest specific activity (enzyme activity/organic C) characterised the most acidic band. The IEF of the (A_I) fraction (with molecular weight higher than 100,000) revealed four enzymatically active bands with about 50% of the total protease activity of fraction concentrated in the band at Ip 4.65.

4.7
Conclusions

In spite of the fact that both clays and humic matrix can adsorb or entrap enzymes, only humic-enzyme complexes have been extracted from soil and characterised (Mayaudon 1986; Nannipieri et al. 1996). Sodium pyrophosphate at neutrality has been used to extract several enzymes from soil. Probably, these enzymes are entrapped in organic complexes surrounded by a network with pores large enough to allow the passage of substrates and reaction products but not that of proteases. This model is not valid for enzymes acting on high molecular weight substrates because if their activity is detected they would also be accessible to proteases (Ladd and Butler 1975). It is noteworthy that the extracted enzymes have not been characterised for their organism origin and thus their precise nature is not known.

A possible relationship between immobilised enzymes, exogenous substrates and soil microorganisms has been hypothesised by Burns (1982). However, this relationship has never been verified because it is still problematic to set up model experiments simulating processes at the microenvironmental scale.

It is now established that also in soil microbiology there is a need to go beyond DNA analysis for better characterization of microbial functions in soil (Nannipieri et al. 2003). The characterisation of both DNA and RNA might allow the determination of the actual species effectively involved in the processes being measured; however, there is a need to measure specific functions and carry out the proteomics approach to better characterise microbial functions. The potential applications of soil proteomics in soil are very promising because we could obtain a better understanding of microbial cell-environment interactions, such as responses of microbial cells to stresses, homologous cell-to-cell interactions, such as quorum sensing,

and heterologous cell-to-cell interactions, such as symbiosis and predation. A correct application of proteomics to soil involves consideration of the complexity of the system; particularly, the fact that only 4% of total N is present in microbial biomass whereas 30–45% of the soil N is present as extracellular stabilised proteins (Stevenson 1986) which cannot be related to microbial activities (Nannipieri et al. 2002). Only proteins associated with microbial cells should be considered for characterising microbial functions in soil and how they respond to changes in agricultural management, pollution, presence of plants, etc. Here this approach has been called *soil functional proteomics* to differentiate it from the characterisation of proteins adsorbed by clay minerals or entrapped by humic molecules (*soil structural proteomics*). Given the large background of stabilised proteins in soil a possible approach can be to separate microbial cells from soil particles followed by the extraction of proteins from microbial cells. Possible drawbacks of the cell extraction method might be the low extraction yields and possible changes in the composition of protein mixture during the extraction procedure. Advantages might be the possibility to use techniques, such as the ICAT technique (Gigy et al. 1999), set up with axenic cultures. Methods based on freeze/thaw and autoclaving have been used to extract proteins from Cd-polluted soil (Singleton et al. 2003) and glomalin from soil (Wright and Upadhyaya 1996, 1998), respectively. Probably, these procedures mainly extract intracellular or ectoproteins, due to cell lysis and due to the low extraction of proteins associated with soil colloids by the buffer solutions used (Nannipieri et al. 1996). However, the addition of a protease inhibitor cocktail is needed because proteases are active during cell lysis in soil, as shown by Renella et al. (2002), and they can hydrolyse the target protein. In addition, contaminants co-extracted with proteins from soil can be present and they should be eliminated by purification procedures before protein analysis. This procedure might cause the loss of specific proteins and/or non-proteic components essential for the function of the target protein. Anyway, each extraction method should also show a recovery yield of known proteins added to soil and extracted by the method used so as to determine the extraction efficiency of the employed procedure. Indeed, the quantitative recovery of the target protein is essential in order to obtain consistent data.

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5 Soil Proteomics: Extraction and Analysis of Proteins from Soils

Oladele A. Ogunseitan

5.1 Introduction

The diversity of organisms in soil habitats is influenced by complex interactions among various physical, chemical, and ecological factors. Microorganisms belonging to all three major phylogenetic domains, namely Archaea, Bacteria, and Eukarya, inhabit soils according to the availability and configuration of niches. These microbes engage in symbiotic, neutral, or pathogenic relationships with large multicellular organisms such as plants. These interactions result in the biogeochemical cycling of several elements that form permanent or transient features of soils. However, these cycles also link soil processes to atmospheric and aquatic environments. Therefore, methods that are developed to investigate structural and functional diversity of soil microorganisms must be sensitive to the fluidic nature of the processes attributed to the organisms and to the possibility of trans-compartmental interactions. However, it has proven difficult to estimate biological diversity in soils because most of the available methods are dependent on cultivation of organisms under laboratory conditions, a notoriously inadequate strategy for accounting for the majority of microbial species. It is for this reason that methodological approaches which focus on molecular signatures have become so powerful since they attempt to resolve questions of structure and function without the need to cultivate organisms on the basis of habitat characteristics (Torsvik et al. 1996, 1998; Nannipieri et al. 2003).

Nucleic acid-based approaches for investigating soil microbial communities rely on the coordinated functions of protein molecules that serve as enzymes for recognising, replicating, and amplifying specific nucleic acid sequences. In addition to this fundamental linkage between protein function and the assessment of genetic diversity, protein molecules represent the final result of genetic expression, and, through their functions as physiological catalysts, structural components, signal transducers, and

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mediators of intercellular communication, they control key reactions in ecological processes performed by microorganisms in soils. The analysis of microbial proteins has traditionally focused on comparative quantitative assessments and functional characteristics of a few phylogenetically conserved molecules. For example, cytochromes, protein elongation factors, and ATPases are among the most widely studied protein molecules for which their genetic coding sequences have been used in systematics (Liu and Stahl 2002). Whereas for ecological functions, most research employing protein-based approaches has focused on enzymes of significance to soil fertility (Nannipieri et al. 2003). However, these previous studies can hardly be called “molecular” because inferences were made without adequate resolution of molecular-scale observations that could facilitate reliable conclusions on system diversity and predictive modeling of responses to variable environmental factors. This chapter focuses on the extraction and analysis of protein molecules as an approach for elucidating structural and functional diversity of soil microbial communities. A survey of methods is presented first, followed by selected case studies on the uses of protein-based methods to solve critical ecological questions. The first case study concerns the production and activities of glomalin by soil microbes. The second case study concerns the extraction of soil proteins to indicate the biological availability of toxic chemicals.

5.2 Rationale and Context of Soil Proteomics

Biogeochemists interested in the nitrogen cycle are credited with the invention of quantitative methods for direct extraction of amino acids and proteins from sediments and soils (Greenfield et al. 1970; Cheng 1975). There is a rather long and detailed historical record of numerous attempts to use semi-qualitative methods for extracting proteins, particularly enzymes, from soil samples. These early techniques were subsequently developed with varying degrees of success to investigate specific enzymes important in agriculture and environmental contamination (Briggs and Segal 1963; Weetall et al. 1965; Burns et al. 1972; Bremner and Zantua 1975; Mayaudon et al. 1975; McLaren et al. 1975; Thornton and McLaren 1975; Pettit et al. 1976; Skujins 1976; Burns 1978; Mayaudon 1986; Nybroe et al. 1990; Tabatabai and Fu 1991; Nannipieri et al. 1996). More recent developments have focused on increasing the throughput of enzyme assays in soil extracts to facilitate rapid assessment of soil conditions and to minimise assay variability due to differences in soil characteristics (e.g. Stemmer 2004).

Soils are extremely complex media in terms of biochemical characteristics. As such, all methods of soil analysis are, in practice, limited to or optimised for a certain fraction of the inherent diversity of organisms and molecules. The total protein fraction of soils represents contributions from prokaryotic and eukaryotic microorganisms as well as from large multicellular eukaryotes and anthropogenic wastes. The portion of the protein fraction that is not immobilised by soil colloids such as clay and humic matter is located in one or more of three major compartments depending on the type and physicochemical conditions of the soil. Intracellular proteins occur in microbial cells as well as animal and plant tissues present in soils. Extracellular protein molecules may exist in the aqueous interphase between particles in moist soils, and this protein fraction results from lysed cells or secreted enzymes and other bioactive proteins. The third soil protein fraction is located on the surface of cells, where the molecules function as exoenzymes active in the transportation of nutrients and growth factors into the cells, or in the active excretion of toxic metabolites out of the cell. Methods that have been developed to assess soil proteins, particularly enzymes, optimise to different extents the recovery of these three fractions. In addition to focusing on specific soil protein fractions, certain methods have also been developed for exhaustive recovery of protein molecules without necessarily preserving activity or secondary structure; on the other hand, other methods strive to preserve protein functions such as catalysis, but, in doing so, the gentle approach of these methods does not achieve complete recovery of proteins present in the soil environment.

Strategies for bulk soil protein assessments are based in part on the rationale that a complete proteome map of a microbial community will facilitate the discovery of unique polypeptides whose production is mediated by specific environmental cues. The molecular resolution of soil proteins for elucidating species diversity and metabolic productivity has proven difficult because of the large number of different proteins synthesised by different species, even in axenic microbial cultures (Blom et al. 1992; Ogunseitan et al. 2001). Despite this limitation, the refinement of immunological techniques coupled with the increasing number of available enzyme assays have been used to supplement the need for detailed molecular resolution in cases where attention is focused on particular polypeptides (Feinstein and Lindahl 1973; Mayaudon and Sarkar 1974; Bohlool and Schmidt 1980; Selander et al. 1986; Wright 1992; Ogunseitan 1993; Paul 1993; Jehr and Hussain 1994). For example, the discovery of glomalin, the complex proteinaceous substance which has been implicated in various structural and functional features of soils, was facilitated by exhaustive soil protein extraction and immunological detection, but the molecular details of this protein remain elusive (Wright and Upadhyaya 1996; Wright et al. 1996; Rillig et al. 2003).

Although the frequency of discoveries in proteomics is increasing, there are major gaps in the state of knowledge linking the presence of specific proteins in complex microbial communities to ecological functions within different fields of investigation. For example, the use of protein sequence information to construct phylogenetic trees has greatly contributed to microbial systematics and the exploration of both quantitative and qualitative aspects of microbial diversity, whereas information on the biochemical ecology of proteins has typically been inferred from axenic cultures lacking the context of multi-species community that enables protein molecules to implement various levels of interactions among organisms (Kersters and De Ley 1980; Jackman 1985; Hantula et al. 1990, 1991; Ogunseitan et al. 2002; Ogunseitan 2005). Therefore, it is questionable whether phenomena discovered under axenic conditions are meaningful in natural ecosystems, but the emergence of a repertoire of methods and techniques that support the analysis of protein synthesis, diversity, and function in natural heterogeneous microbial communities is enhancing research towards realistic environmental proteomics (Nannipieri et al. 1974; Busto et al. 1995; Ogunseitan 1997, 1998).

Elucidating ecosystem-level activities by assessing molecular diversity in soil environments demands an awareness of the complexity of protein structures when compared to other signature biomolecules such as nucleic acids and fatty acids. Protein complexity increases the richness of information extractable from an environmental sample, but the available techniques for analysing the information are not only few, but also complicated by many layers of uncertainty. The fundamental level of information contained in protein molecules is represented by the primary structure, or the sequence of ∼20 possible amino acids making up the polypeptide chain. Comparative protein profiles based on physical size and electric charge properties can be routinely constructed through one- or two-dimensional polyacrylamide gel electrophoreses (PAGE) which are readily available for studies focused on temporal and spatial variations in genetic induction of protein synthesis. Amino acid sequence analysis can also address questions of evolutionary diversity among specific groups of organisms occupying similar ecological niches. However, protein function also depends on its secondary (folded protein), tertiary (globular protein), or quaternary (several interacting folded polypeptides) forms. These morphological conformations contribute significantly to the ability of proteins to perform crucial functions such as enzymatic catalysis and organelle construction. The abundance and diversity of microbial proteins suggest fairly straightforward extraction methods, but attention must be paid to extraction conditions that preserve protein integrity and function, while reducing interference from co-extracted substances such as nucleic acids and humic materials. It is particularly important that functional enzyme

studies employ extraction, resolution, and detection techniques that optimise the stability of protein conformation while minimising interference by potential inhibitors such as metal ions and detergents which act as denaturing agents.

The rationale for direct extraction of proteins from soils and other environmental samples includes monitoring the activities of particular enzymes without necessarily identifying specific organism sources (Ogunseitan 1998; Ogunseitan et al. 2000); monitoring the fate of genetically engineered organisms that produce commercially desirable protein molecules such as the *Bacillus thuringiensis* crystal toxin (Saxena et al. 2002; Lee et al. 2003); detection of specific antigens associated with non-culturable bacteria or pathogenic virus particles in soil (Vettori et al. 2000); and characterisation of the response of microorganisms to stressful physical-chemical environmental conditions (Ogunseitan 1996, 1999). In the following sections, techniques useful for extracting proteins from complex microbial communities are described with emphasis on the preservation of enzymatic activities. New techniques, including protein chip arrays, are described briefly because they offer high resolving power, although their application has thus far principally been limited to the study of pure cultures and simple assemblages of microbial populations.

5.3 Methodology for Soil Proteomics

Methods for extracting protein molecules from soils are entirely independent of the subsequent methods of protein analysis. If the analytical methods involve assessment of protein function, including enzyme activity, then the extraction methods must preserve function as much as possible. For example, the availability of colorimetric staining techniques for more than 300 enzymes (Manchenko 1994) coupled with nitrocellulose membrane-immobilisation of protein molecules extracted from environmental samples has aided rapid screening of multiple enzymes from a single source, thereby facilitating comparative analysis of specific metabolic activities across samples (Ogunseitan et al. 2000, 2001, 2002). Alternatively, if analytical methods are focused on molecular size comparisons or immunological detection, then extraction methods are generally less conservative.

5.3.1 Extraction Methods

All protein extraction methods aim to achieve quantitative recovery of polypeptides from the complex matrix of soil particles, and to purify the

extract as much as possible by removing unwanted molecules that may interfere with further analysis. However, many protein molecules exist in nature as complexes with other kinds of molecules, including colloids, nucleoproteins, glycoproteins, and metalloproteins. In some cases, these complexes interfere with protein detection and quantification techniques, and, importantly, methods which aim at detecting protein function should not strip polypeptides of the essential non-proteinous components that are essential for optimum activity.

Sonication is one of the most common methods for breaking apart aggregated soil particles and for lysing cells to release protein molecules. However, sonication may also cause the denaturation of proteins (sometimes visible as foam) under certain conditions, depending on soil characteristics; but working at low temperatures ameliorates this detrimental effect. Following sonication, centrifugation is typically used to recover protein fractions which may be further purified by dialysis or salt precipitation (Ogunseitan and LeBlanc 2004). In certain cases, "soil washing" is used to first release cells from the soil matrix prior to protein extraction, although this step introduces another level of uncertainty that compromises the exhaustive recovery of the protein content of soils (Lindahl and Bakken 1995).

Many techniques have been published for extracting protein molecules from bulk soil, and some case-by-case evaluation is still necessary. In the simplest case, briefly, the extraction of proteins from bulk soils usually involves the suspension of 1–100 g of fresh sample in 1.5 volumes (w/v) of cold 0.3 M K_2HPO_4, plus 0.5 volume of 0.3 M EDTA (pH 8.0; Singleton et al. 2003; Harner et al. 2004; Ogunseitan and LeBlanc 2004). The suspension is subjected to sonication using a medium-tipped probe such as the 1 cm tip accompanying the sonic dismembrator model 550 (Fisher Scientific, Tustin, CA, USA) at ~20 kHz for 30–60 min (in 10 s pulses) in an ice bucket. The samples are then agitated on a rotary shaker at 100 rpm in a cold room (0–4 °C) for 4 h to overnight. To recover the protein fraction, the samples are centrifuged at 3,000 *g* for 10 min to remove large particles. The supernatant is collected for repeated centrifugation at 25,000 *g* for 25 min at 4 °C to completely remove all suspended particulate matter. Protein molecules can be precipitated from the supernatant by adding 60% (w/v) $(NH_4)_2SO_4$, adjusting the pH to 7.0 by adding 1 M KH_2PO_4, and incubating at 0–4 °C for 2–6 h. Precipitated proteins are then recovered by centrifugation at 25,000 *g* for 25 min at 4 °C, followed by resuspension in an appropriate volume (e.g. 10 ml) of 0.2 M K_2HPO_4 per gram of wet precipitate, with adjustment of pH to 7.0.

Further purification of soil protein extracts can be achieved by dialysis against 0.02 M phosphate buffer overnight in a cold room (0–4 °C; Ogunseitan 1993). The dialysate is centrifuged at 25,000 *g* for 25 min at 4 °C to

collect supernatant which is now ready for the determination of protein concentration, enzyme assays, electrophoresis, and other analytical methods (Schulze et al. 2005). Soil protein extracts may also be filtered through a 0.2 μm Nalgene syringe filter (Nalge, Rochester, NY, USA) and stored at −20 °C until further analysis.

Several variations to the general protocol described in the previous paragraphs have been reported in the literature, with the most notable alternatives to sonication being the "freeze/thaw", "bead-beating", and "autoclaving" methods (Wright and Upadhyaya 1996; Bolygo et al. 2003; Barzaghi et al. 2004). The former two methods begin by suspending samples of soil (e.g. 1 g at 50% water-holding capacity) in 1 ml of an extraction buffer consisting of 50 mM Tris-HCl, 10% sucrose, 2 mM dithiothreitol, 4 mM EDTA, and 0.1% Brij 58. The final pH of the buffer is adjusted to 7.5 with ammonia solution, and 100 μl of protease inhibitor cocktail (e.g. Sigma P2714) are added to the sample plus buffer (Eprogen Corporation 2004). For the freeze/thaw approach, the soil suspension is vortexed briefly (10 s) and subjected to four cycles of freezing in liquid nitrogen and thawing at 25 °C in a vented container (Ogunseitan 1993). The supernatant is then collected by sequential centrifugation to ensure the removal of all soil particles and cell debris. Sample preparation is similar for the bead-beating approach except that, instead of freeze/thawing, the soil suspension is shaken at 5.5 m s^{-1} for 30 s in a vessel containing 0.2 g of sterile glass beads (150–212 μm diameter available from Sigma, St. Louis, MO, USA). Both the freeze/thaw and bead-beating approaches have been used to monitor the response of soil proteins to toxic pollutants (Singleton et al. 2003).

The autoclaving approach has been used most notably in the recovery of abundant recalcitrant proteins such as glomalin from soil (Wright and Upadhyaya 1996, 1998; Rillig et al. 2002a). For example, total soil glomalin can be extracted by suspending 1 g of soil samples in 8 ml of an extraction buffer consisting of 50 mM citrate at pH 8.0 and autoclaving this mixture at 121 °C for 60 min. The supernatant is then recovered by centrifugation at 5,000 *g* for 20 min. The autoclaving and centrifugation cycle is repeated for as many times as necessary until the supernatant is clear of the brown discolouration attributable to glomalin. A final centrifugation at 10,000 *g* is conducted to remove all soil particles and cell debris.

5.3.2 Analytical Methods

The reliability of inferences about the presence or absence of specific polypeptides in a soil protein extract depends not only on the rigour of the extraction procedure, but also on the accuracy of the technique used to

estimate the concentration of protein molecules in the extract, and on the sensitivity of the analytical technique used for protein detection. For most purposes, the determination of protein concentration in a given extract can be achieved through spectrophotometric analysis using various dyes (e.g. Bradford Dye assay; US Biochemicals, Cleveland, OH, USA). The sensitivity of the protein assay can be improved by incubating the dye reaction at 37 °C for 1 h prior to spectrophotometer readings (Bradford 1976; Zor and Selinger 1996). If necessary, protein molecules can be concentrated further in the extract by centrifugation at 2,000 *g* for 10 min in a Centriprep cartridge with a 3 kDa molecular weight cut-off (Ogunseitan and LeBlanc 2004).

Protein Profiling by Electrophoretic Resolution

PAGE in one or two dimensions is a relatively straightforward and quick way of assessing the diversity of polypeptides in a protein extract, although the resolving power and visualisation of protein bands depend on the staining protocol (typically silver-based or Coomassie brilliant blue is used); on the level of interference by the presence of contaminants which may produce low signal-to-background ratios; and on the level of sophistication of software used for comparative assessment of polypeptide banding patterns within gels and across electrophoretic runs (Ogunseitan 1993, 1999; Ogunseitan et al. 2001; Singleton et al. 2003). In cases where specific experiments allow the use of radioactive tracers, the identity of newly synthesised proteins in a microbial community can be determined by autoradiographical analysis of gels used to resolve complex protein extracts (Ogunseitan 1996). In these cases, radioisotopes are typically added to soil microcosms in the form of inorganic sulphate or labelled methionine. Alternately, depending on the objectives of a given experiment, radioactive substrates may be provided to monitor the outcome of catabolic reactions conducted by specific enzymes.

Enzyme Assays

When enzymes are targeted for monitoring, starch gel electrophoresis is useful for evaluating protein extracts for immobilised enzyme activities (Ogunseitan 1997). Enzyme assays can also be performed on nitrocellulose membranes, or ultrathin polyacrylamide gels (Jehr and Hussain 1994; Ogunseitan 1998; Ogunseitan 2002). Based on these approaches, it is conceivable that if sufficient level of purity is achieved, soil protein extracts can eventually be used for multilocus enzyme electrophoresis (MLEE) to reveal polymorphisms at specific genetic loci in the various populations of a microbial community (Shaw and Prasad 1970; Selander et al. 1986). These polymorphisms are expressed as redundancies in metabolic capac-

ity which contributes to the resilience of ecological functions in stressful environments. For example, the occurrence and distribution of various forms of enzymes which express the same activity but differ in amino acid sequence (allozymes are produced by different alleles at a single locus) can reveal meaningful information on evolutionary divergence of different population members which colonise the same niches in an ecological community. Such analyses have been conducted for aquatic systems (Ogunseitan 1999; Ogunseitan et al. 2002). The advantage of using nitrocellulose membranes in a slot-blot apparatus is that larger volumes of protein extract can be immobilised than when using gel-based techniques. However, with the membrane technique it is not possible to detect enzyme polymorphisms at the molecular level. Soil protein extracts can also be used for immunological detection of specific antigens (Wright 1992, 1994).

Microarray Analysis

Rapid progress in genome sequencing efforts has engendered technological advances in bioinformatics through the accumulations of large databases aimed at unraveling the linkages between genetic potential and phenotypes. One of the major developments in this direction is the production of DNA microarrays for functional genomics analysis of gene expression. Similarly, innovations in instrumentation and experimental techniques have provided new ways for analysing the protein content of cells through differential display arrays (Barzaghi et al. 2004). Such innovations have greatly facilitated our ability to discover polypeptides that mediate cellular responses to various environmental stimuli, although major challenges still exist in applying these innovations to complex systems such as soils. Recently, the development of microarray systems based on miniature chips and mass spectrometry has facilitated the analysis of large numbers of protein molecules, under different experimental conditions, to provide relatively precise data on protein profiles (Ogunseitan et al. 2001; Schulze et al. 2005). The 2002 Nobel Prize in Chemistry was awarded jointly to John Fenn, Koichi Tanaka, and Kurt Wüthrich for developing sophisticated instrumental and methodological approaches for analysing proteins, including spectrometry and magnetic resonance imaging. These achievements have enhanced the recognition that proteome-based strategies will provide significant contributions in the post-genomic research era (Wüthrich 2001; The Nobel Prize in Chemistry 2002).

Technologies for proteome analysis are intrinsically more complex than those used for nucleic acid analysis. Although this is partly due to the larger number of amino acids (20) as compared to the number of nucleic acid bases (5), the greatest complication comes from the post-translational modifications which endow each category of polypeptide chain with a unique

set of characteristics (Smith et al. 2002). Microarray systems combine multiple technologies in a unit platform that facilitates protein immobilisation, purification, analysis, and processing from complex biological mixtures. In particular, the development of surface-enhanced laser desorption/ionisation time-of-flight (SELDI-TOF) mass analysis has increased experimental options for differential display analysis of proteomes for organisms cultivated under different environmental conditions. Developments in coupled mass spectrometric analysis of proteomes in microarray formats provide new opportunities for ecologically relevant soil proteome assessment, including spatial-temporal and quantitative mapping of protein involvement in organism–environment interactions, and in cell–cell communication (Ogunseitan et al. 2002; Ogunseitan and LeBlanc 2004).

The SELDI-TOF process has four major components. The first component requires the capturing of target molecules from the protein extract onto the microarray with minimal sample preparation. The second component involves the enhancement of the signal-to-background ratio through the use of selective “washing” protocols. The third component deals with the identification of remainder protein molecules on the array by means of a laser-induced process to generate a protein mass profile. Finally, target protein molecules can be characterised directly through modification reactions that provide information about protein structure and function. The information gained can thus be verified through comparison to an extensive database of protein sequence, size, conformation, and activity to identify known proteins or characterise entirely novel discoveries. In general, the arrays contain chemically treated surfaces that facilitate specific interactions with protein molecules, including cationic, anionic, hydrophobic, and hydrophilic reactions. Similarly, arrays may be biochemically configured with specific antibodies, cell-surface receptors, or nucleic acids, to identify heterogeneous molecular interactions (Ogunseitan and LeBlanc 2004). Here again, it is important to emphasise that successful application of these approaches depends on the quality of protein extracts derived from soils. Different soil types will invariably require different protein purification strategies to remove contaminants and co-extracting biochemicals that may interfere with molecular resolution and quantification procedures.

5.4 Case Studies and Emerging Issues in Soil Proteomics

The appeal of molecular techniques in soil science is attributable to their power in dissecting complex features to reveal component structures and functions that facilitate better understanding of how these components are interrelated; and how the relationships can be exploited to craft sustain-

able solutions to persistent environmental problems. This section focuses on two case studies where the use of protein extraction and analysis methods has led to new understanding of soil properties and how soil organisms cope with stressful conditions. The first case explores the role of glomalin in creating the features that support soil particle aggregation and carbon (C) storage. The second case involves the role of protein extraction and analytical methods in understanding how soil microbial communities respond to pollutants such as toxic metals. These cases are selected as possible extremes out of several possible general cases where protein analyses in soils have been successfully achieved because of the strengths of the lessons they bear for innovation in the midst of challenging methodology.

5.4.1 Glomalin

In 1996, Sara Wright and colleagues discovered an abundant soil protein that has been associated with several important soil properties and biogeochemical functions (Wright and Upadhyaya 1996). The protein, named glomalin for its association with arbuscular mycorrhizal fungi belonging to the order *Glomales*, is extremely stable in soils where it accumulates to several mg per g of soil, reaching up to 100 mg per g in some Hawaiian soils (Fig. 5.1). Two fractions of glomalin are known to be present in soils: the easily extractable glomalin (EEG), and total extractable glomalin (TEG). The extraction of glomalin from soils requires the unusual step of autoclaving. Typically for EEG, 1 g of soil is extracted with 8 ml aliquots of 20 mM sodium citrate under autoclaving conditions (15 psi pressure and 121 °C) for 30 min. For TEG, 50 mM sodium citrate is used, and the autoclaving time is doubled. It is noteworthy that citrate will also cause the co-extraction of other non-proteinous soil components that may interfere with the quantitative analysis of glomalin further on in the process. The extraction is repeated as long as no more brown-coloured glomalin is visible in the remaining particulate soil fraction. Centrifugation-clarified extracts of glomalin are stable as freeze-dried protein (Fig. 5.2).

Glomalin is known to be associated with carbohydrates (glycoproteinous) containing between 30–40% C by weight, and 1–9% of tightly bound iron, but, beyond that, not much else is known about its structure (Gonzalez-Chavez et al. 2004). In addition, neither the environmental cues for the production of glomalin nor its specific cellular biological role and processing in the fungi that produce it are well understood. However, because it is detectable by means of immunological techniques, several studies have demonstrated the ubiquitous occurrence of glomalin in soils

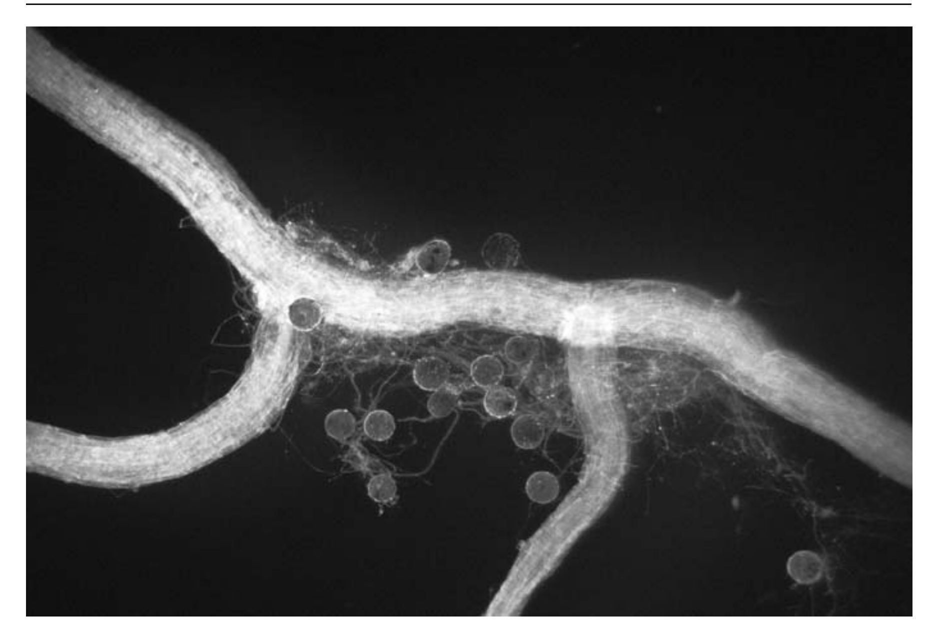


Fig. 5.1. View of glomalin revealed by immunological reactions using a green dye tagged to an antibody against glomalin on an arbuscular mycorrhizal fungus growing on a corn root. The *round structures* are spores, and the *threadlike filaments* are hyphae. Photograph by Sara Wright, United States Department of Agriculture

across many geographical zones and land-use patterns (Wright and Upadhyaya 1998; Rillig et al. 2002b; Knorr et al. 2003; Steinberg and Rillig 2003).

Several interesting ecological roles have been attributed to glomalin. These ecological functions include its role as "glue" between soil particles thereby explaining many features regarding soil texture, soil aggregation, water-holding capacity, autochthonous microbial diversity, and the stability or turnover rate of the soil C pool. Glomalin concentrations in soil also correlate with land-use activities, and differ between tilled or agricultural soils and forested regions, and between tropical and temperate climate soils (Rillig et al. 2003). Glomalin has been shown to account for a great proportion of the organic C in soils. Therefore, this protein is also a major component of soil organic matter, weighing 2 to 24 times more than humic acid, which was previously thought to be the main contributor (approximately 8%) to soil C. Rillig and colleagues (2003) have estimated that glomalin molecules survive in soils for 7–42 years, depending on soil temperature, humidity conditions, and other physicochemical properties of soils. Apparently, higher levels of atmospheric carbon dioxide stimulate the growth of glomalin-producing fungi, and consequently the level of glo-



Fig. 5.2. Freeze-dried glomalin extracted from undisturbed soil, where it is present in concentrations of several milligrams per gram of soil. Photograph by Keith Weller, United States Department of Agriculture

malin found in soils. This and other observations have raised the profile of glomalin management in soils as a potential indicator of the ecological effects of global climate change (Rillig et al. 2002a).

Apparently much remains to be learned about the molecular details of the glomalin structure, the diversity of organisms capable of producing glomalin, and the diversity of organisms capable of degrading the protein. These challenges will require the orchestration of a wide variety of the proteomics techniques discussed in the first sections of this chapter. In particular, glomalin extracts need to be subjected to molecular mass fingerprinting techniques such as the protein-chip system based on SELDI-TOF

technology. This approach could reveal the fine-scale molecular diversity expected by the differences in the extraction procedure attributable to the easily extractable and total extractable glomalin.

5.4.2 Soil Proteins as Metal Biosensors

It has been estimated that at least 30% of all proteins are metalloproteins, requiring one or more metal ions for proper functioning (Andreini et al. 2004; for examples, see Table 5.1). The specificity of protein–metal complexes and the affinity (and stoichiometric interaction) of proteins for metals in general provide an opportunity to detect or to determine biological availability of metal ions by assaying protein functions including enzymatic activity (Haraguchi 2004). Theoretically, two major approaches are possible for developing protein-based strategies for indicating the biological availability of metallic contaminants in soil. The first approach relies on the comparative assessment of the profiles of polypeptide molecules extracted directly from soils in the presence and absence of the pollutant in question. The robustness of such differential displays depends on pattern recognition techniques, and is often difficult to interpret because of the labile nature of protein expression in organisms. The second, rather traditional approach is more narrowly defined, seeking to detect changes in the expression and activity of a particular soil protein in response to metal exposure (Tyler 1974). For example, it is possible to correlate delta-aminolevulinate dehydratase activity with the concentration of biologically available lead (Pb) in contaminated systems (Ogunseitan et al. 2000).

As described in Sect. 5.4.1, direct extraction methods have been used to investigate the contribution of extracellular fungal proteins to soil stabilisation (Wright and Upadhyaya 1996). Yields between 1 and 100 mg protein per g of soil have been reported by investigators employing prolonged heat extraction to generate proteins detectable by enzyme-linked immunosorbent assay (ELISA) from agricultural soils (Rillig et al. 2003). Conversely, methods aimed at protecting protein function clearly cannot employ the autoclave, and consequently these methods typically achieve lower protein yields. In situations where a specific group of proteins is targeted, it is worthwhile to use extraction conditions such as buffer-pH and temperature that maximise protein stability, although it must be emphasised that most extraction techniques are only relatively successful with respect to soil chemistry and biological composition. Consequently, decisions about trade-offs must be made between optimising extraction effectiveness and representativeness. For example, in vitro studies have used deoxycholate to extract ribosomal proteins, and urea pre-extraction has been used for

Table 5.1. Examples of metals required by key enzyme functions. The biological availability of metal ions affects enzyme function, potentially constituting the basis for the development of protein-based biosensors

Metal	Enzyme examples	Relevant organisms and ecologicalfunction
Calcium	Collagenase Calpain protease	*Clostridium histolyticum* Pathogenesis
Cobalt	Halomethane methyltransferase	Facultative methylotrophs Chloromethane degradation
Copper	Copper amine oxidases	*Arthrobacter globoformis* Deamination of primary amines to corresponding aldehydes
Iron	Cytochrome P450 Soluble Methane Monooxygenase	*Pseudomonas putida.* P450cam Polyaromatic hydrocarbon biodegradation
Magnesium	Magnesium chelatase	*Rhodobacter sphaeroides* Chlorophyll synthesis
Manganese	Manganese-dependent peroxidases	*Phanerochaete chrysosporium* Carbon cycling/lignin degradation
Molybdenum	Xanthine dehydrogenase Nitrate reductase; nitrogenase	*Pseudomonas putida* *Rhizobium* Purine (caffeine) degradation Nitrogen fixation
Selenium	Selenocysteine (UGA codon) enzymes; glutathione peroxidase; hydrogenases	*Escherichia coli* *Methanococcus voltae* Nitrogen respiration
Tungsten	"True W-enzymes" Aldehyde ferredoxin oxidoreductase	*Thermophilic Archaea*; *Pyrococcus furiosus* Molybdenum antagonist
Zinc	Aminolevulinate dehydratase (inhibited by Pb)	Several bacteria and nearly all archaea Porphyrin synthesis

bacterial membrane proteins (Deutscher 1990). Systematic studies of such protein fractions, particularly for enzymes in natural environments, have yet to be standardised, but certainly represent a field in microbial ecology that is ripe for further investigation.

Singleton and colleagues (2003) successfully demonstrated that the presence of cadmium (Cd) in soils affects both the concentration of total proteins and the size distribution of proteins extracted from soils. In this regard, soil protein content was found to be as sensitive as measurements of soil microbial biomass in indicating soil ecosystem stress from toxic metals (Sandaa et al. 2001). The increase in the production of small molecular weight proteins (< 21 kDa) in response to Cd exposure may be related

to the observation in laboratory cultures that organisms synthesise metallothionein and similar proteins as a protective measure against metal toxicity. However, a validation of the putative correlation between bulk protein expression and metal concentration will require further research. It is quite possible that such correlations will prove difficult to establish with confidence given the complex variability of physical and chemical parameters of soils. In such cases, establishing and integrating the responses of several key individual soil organisms may provide a feasible strategy for approximating community-level responses. However, challenges posed by the enormity of the statistical processing capacity needed for such computations may prove insurmountable. Instead, a compartmentalised approach where pools of active and inactive microbial populations are evaluated to derive approximations of overall soil processes is perhaps inevitable.

We have used the SELDI-TOF technology to investigate the response of individual microbial protein profiles to toxic metal exposure (Ogunseitan et al. 2002). We focused specifically on mercury and lead because these metals are not only capable of inducing genetic expression, but they also have the capacity to destabilise cellular metabolism by inhibiting the functions of several enzymes (Ogunseitan et al. 2000). Specific heavy metal resistance systems have evolved in the microbial communities (Ogunseitan et al. 1999), but several observations have also demonstrated that genetically encoded metal resistance is not found in all organisms inhabiting a metal-contaminated ecosystem (Ogunseitan 1998). Metal-resistant organisms typically exhibit a concentration threshold depending on whether the resistance mechanism is based on limiting metal ion transportation from outside to inside the cell, or on enzymatic transformation of metals after active transportation into the cell, as mediated by mercuric reductase in the case of mercuric ions (Ogunseitan 1998). These ecological contingencies on microbial interactions with toxic metals in the environment provide a rich case study for analysing the whole proteome in order to understand how organisms cope with environmental stress at the molecular level.

5.4.3
Prospects for Proteomic Analysis of Soil Microbial Communities

Research in the newly emerging field of soil proteomics proteins has aligned with three important areas of investigation. The most active of these is focused on cell–environment interactions, including protein-based biosensors, ectoenzyme activities, stress proteins, and the induction of metabolic proteins by fluctuating concentrations of environmental chemicals. The second area involves the analysis of homologous cell-to-cell interactions, including quorum sensing, genetic exchange activities, and secondary

structure or colony formation. The third area of focus is the investigation of heterologous cell-to-cell interactions, involving the production of bioactive peptides that define competition, predation, commensalisms, and symbiosis in the natural microbial community (Ogunseitan et al. 2002).

Although great strides are being made in these areas of focus within microbial environmental proteomics research, there are three major challenges for future developments toward the analysis of soil microbial community functions through protein assessment. The first challenge concerns the exhaustive recovery of proteins from heterogeneous populations inhabiting proximate habitats. Traditionally, protein extraction methods have been developed to optimise the selective recovery of active proteins through non-destructive methods such as sonication or to maximise the recovery of total proteins by harsh cell lysis conditions such as heat treatment with detergents. In addition, methods have been developed to recover extracellular proteins through soils washing, and the use of differential centrifugation, thereby avoiding recovery of particulate proteins (Ogunseitan 1997). A complete proteome assessment should rely on methodological integration that requires the optimisation of protein recovery while preserving functional characteristics. There is no doubt that trade-offs must be made between these two objectives, depending on particular soil characteristics and the stated goal of research topics.

The second challenge concerns the resolution of independent protein molecules extracted from microbial populations in a manner that accommodates the dynamic nature of gene expression (including polymorphisms), protein synthesis and protein degradation. The use of matrix-assisted laser desorption or electrospray ionisation mass spectrometry has achieved enhanced resolution, and, theoretically, up to 30,000 polypeptides can be systematically resolved through these techniques. However, given the size of microbial genomes and the very high diversity that exists in most soil microbial communities, only a small fraction of existing polypeptides can be resolved even by these high-throughput techniques. The methodological matrix of resolving conditions associated with SELDI-TOF can assist in a stepwise protein resolution scheme that will increase the coverage and resolving power of community proteomes, but integrating the data requires extensive computing and numerous controls. Thus, the third challenge lies in the development of new bio-informatics techniques for processing large databases of protein mass and function data. Neural networks developed for pattern recognition of protein profiles show good promise in this direction (Ogunseitan 2002).

Ultimately, as in other fields of molecular biology, there is reasonable optimism that technical developments will catch up with the rapid pace at which key research questions are being framed within soil science that requires the application of proteomics. This chapter explored recent

methodological developments and salient case studies. These and other path-defining explorations in soil proteomics will continue to contribute significantly to our understanding of phenomena such as soil structure, biodiversity, and biogeochemical cycling, including the response of soil systems to toxic pollutants.

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6 The Various Sources and the Fate of Nucleic Acids in Soil

Wilfried Wackernagel

6.1 Introduction

DNA is the genetic material of all cellular organisms living in the biosphere. Thus, DNA is expected to be present in any habitat of prokaryotic and eukaryotic organisms. The DNA may be either contained within living or dead cells or may exist as extracellular DNA released from organisms during life or death. In addition, DNA may be disseminated by bacteriophages and viruses. This chapter focuses on the fate of DNA in soil habitats. Among biological macromolecules, DNA is the only informational entity which after release from cells can actively be taken up by bacteria and its information used in a process termed natural genetic transformation (see Chap. 15). During the past decades the genetic engineering methodology has been used to introduce specific DNA constructs into numerous organisms and with these often purposely into the environment. Among the genetically engineered organisms released into the environment in field studies and during commercial use are agricultural plants including transgenic plant varieties of potato, tobacco, maize, sugar beet, rape, soy bean, cotton, rice, and others (Brandt 2003); microorganisms including species of *Pseudomonas* and *Rhizobium* (Drahos et al. 1988; Lindow and Panopoulos 1988; Stewart-Tull and Sussman 1992; Selbitschka et al. 1995); and viruses like Baculovirus (Bishop et al. 1988). The recombinant DNA constructs are considered to be unique since the fused DNA sequences which are normally not present in nature provide the chance for a highly specific and sensitive DNA monitoring in environmental samples because of the absence of a natural background. The detection of specific sequence fusions can be accomplished by polymerase chain reaction (PCR) and novel biomonitoring techniques (Recorbet et al. 1993; Widmer et al. 1997; de Vries and Wackernagel 1998; Paget et al. 1998; Gebhard and Smalla 1999; de Vries et al. 2003; Meier and Wackernagel 2003a,b). This chapter summarises experiments and observations aimed at clarifying the question of how DNA is released

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from organisms and what the fate of extracellular DNA is with emphasis on the particular situation in soils. Soils are complex physicochemical matrices consisting of solid, liquid and gaseous phases with variable contents of inorganic and organic matter (for details, see Chap. 2). Soils and sediments are the habitats of uncountable numbers of species from all kingdoms.

6.2 Release of DNA from Organisms

The organisms living in soil encompass mainly prokaryotes, fungi, and various animals. Plants can grow their roots in soil and often also rhizomes and parts of the shoot. In order to obtain clues as to the presence of extracellular DNA in the environment including soil, the release of DNA from members of these groups has been examined. The experiments were mainly performed in the laboratory and only in few cases in natural field soils or in soil microcosms.

6.2.1 Bacteria

The soil bacterium *Bacillus subtilis* releases DNA during growth in broth cultures in temporal coincidence with competence development (Sinha and Iyer 1971; Crabb et al. 1977; Lorenz et al. 1991). A particularly strong DNA release occurs during stationary and death phase when the cell wall associated autolysines start to destabilise the cell wall (Svarachorn et al. 1989). The effects of nutrient limitation and low temperatures can potentiate the DNA release by lysis (Svarachorn et al. 1991). The supernatants of other soil bacterial cultures including *Azotobacter*, *Alcaligenes*, *Pseudomonas*, *Synechocystis*, *Acinetobacter*, and *Micrococcus* species all contain high molecular weight fragments of chromosomal DNA (for references, see Lorenz and Wackernagel 1994). Released DNA including intact plasmids was functional as shown by its transforming activity for competent recipient cells, e.g. of *B. subtilis* (Lorenz et al. 1991) and *Acinetobacter* (Lorenz et al. 1991; Palmen and Hellingwerf 1995). The DNA released by lysis appears to be naked, whereas DNA from a number of Gram-negative bacteria including the soil bacterium *Agrobacterium tumefaciens* can be released packaged in membrane-derived vesicles termed blebs (Dorward and Garon 1990).

Whether death of bacteria in the soil habitat is accompanied by lysis and DNA release is not clear for most of the above-mentioned soil bacteria. It is possible that in situ DNA is degraded by DNases within the dead cells.

This question has been addressed by introducing *Escherichia coli* K12 cells harbouring plasmid p707 into different natural soils. The results are shown in Fig. 6.1 (Blum et al. 1997). A continual loss of the viable counts and the number of plasmid targets (determined by quantitative PCR) was followed over a period of 10 days. While the titre of the soil endogenous colony-forming units remained constant, the survival of *E. coli* decreased by 3 to 4 logs. The numbers of p707 targets present in the total DNA extracted from the soils also decreased during the 10 days in soil. However, the number of p707 targets recovered from soil as free extracellular DNA was very low at the beginning of the incubation, but increased 4 to 20 times during the time span when 95% of the inoculated *E. coli* cells had lost colony-forming potential. The data in Fig. 6.1 show that during death of cells much of the intracellular DNA is degraded, but that at the same time a considerable fraction is released into the soil and can associate with soil solids from which this DNA can be recovered (by a specific protocol, see Blum et al. 1997).

Natural conditions which can increase DNA release from bacteria within soil are cycles of freezing and thawing (Tsai and Olsen 1991; Sikorski et al. 1998) and mechanical forces like mixing or grinding of dry soil (Frostegård et al. 1999). An important biotic factor for the release of bacterial DNA may be the grazing of protozoa (Stotzky 1986; England et al. 1993). It was shown that nanoflagellates release much of the DNA of ingested bacteria into the environment (Turk et al. 1992). Also, infection of bacteria by phages with concomitant cell lysis may contribute to the DNA release particularly during seasonal variation of viral concentrations. Although studies related to this topic were conducted in aquatic environments (Proctor and Fuhrman 1990; Börsheim 1993), it is quite likely that in soil ecosystems a similar dynamic balance between bacterial and the cognate phage populations provides a periodical release of bacterial DNA.

6.2.2
Plants

It is expected that DNA from plant tissue will be emitted into soil, because root material as well as leaves and shoot material are deposited in soil or on the soil surface during growth of plants and their senescence. The recombinant genetic constructs of transgenic plants allow the specific identification of plant DNA by highly sensitive PCR assays targeting at the junctions of sequences normally not contiguous in nature (Widmer et al. 1996, 1997; Paget et al. 1998; Gebhard and Smalla 1999; de Vries et al. 2003; Meier and Wackernagel 2003a) and thereby have helped to trace the DNA from the plants. These tests were recently complemented by biomonitoring methods

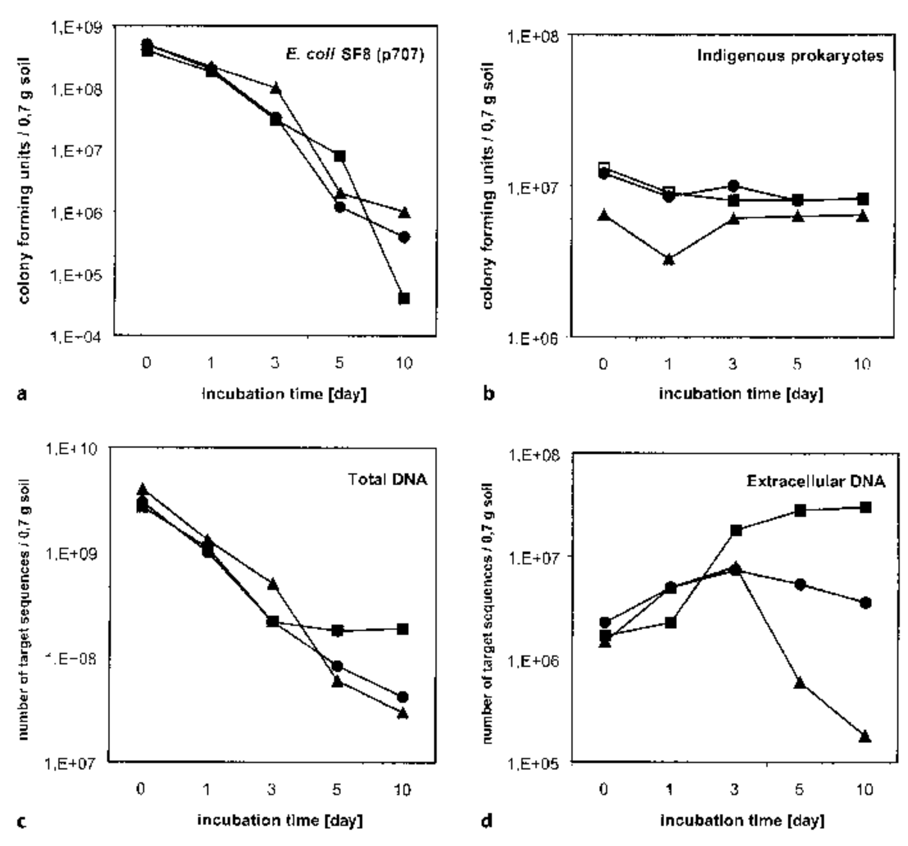


Fig. 6.1. Release of DNA from *E. coli* cells during their death phase in three non-sterile soils (● brown earth, classified as loamy sand; ■ loamy soil, classified as sandy loam; ▲ podzol, classified as sand). Microcosms (Blum et al. 1997) were filled with soil, loaded with washed cells (5×10^8 per 0.7 g of soil) of *E. coli* SF8 (Graupner and Wackernagel 1996) harbouring plasmid p707 (Tebbe and Vahjen 1993; the plasmid carries the genes for β-lactamase and the human aprotinin) and incubated at 23 °C. **a** Survival of *E. coli*; cells recovered from soil were plated on LB medium with cycloheximide (50 µg/ml) and ampicillin (100 µg/ml) at 37 °C and counted after 24 h. **b** Colony-forming units of indigenous prokaryotes were determined on 0.25 × YETG plates (Blum et al. 1997) with cycloheximide (50 µg/ml) after 3 days at 23 °C. **c** Total DNA was extracted from soil by a procedure involving hot SDS and guanidine thiocyanate (followed by purification by ion-exchange chromatography) and the number of aprotinin target genes was determined by quantitative PCR (Romanowski et al. 1993b) of a 390 bp sequence using primers ALF1 (5′-CGC AGC ATC CTC CGC ATT AG-3′) and APR7 (5′-AGC ACC ACC GCA AGT ACG CA-3′). **d** Extracellular DNA was recovered by elution of the soil with TN588 buffer (Blum et al. 1997) and the number of aprotinin target sequences determined as in (**c**). For each time point and each type of measurement (**a**–**d**) three microcosms were sacrificed. The mean of the results is reported

(see Sect. 6.5.3) in which the DNA present in soil or extracted from soil was used to naturally transform competent "indicator" bacteria. In this way, the specific tracing of transgenic plant DNA in soil was possible (de Vries et al. 2003; Meier and Wackernagel 2003a). In most studies the DNA was extracted from environmental samples by harsh procedures which break cells (extraction of total DNA). In some studies mild DNA recovery techniques with environmental samples were employed which avoided the breakage of cells and therefore provided access to the extracellular DNA in soil (see Sect. 6.3). In this way DNA was detected in the aqueous extracts of rhizosphere material of transgenic potato plants throughout the vegetative period (de Vries et al. 2003) and was also found during the growth season in aqueous elutes of soil from field plots with transgenic sugar beets (Meier and Wackernagel 2003a). These observations suggest that during growth of roots in soil a release of nuclear DNA into the soil matrix occurs, e.g. from decaying root calyptra cells or spontaneously broken root hair cells. In both studies the recombinant DNA was monitored by PCR amplification and also by natural transformation of competent soil bacteria. These were *Pseudomonas stutzeri* pMR7 (Meier and Wackernagel 2003a) and *Acinetobacter* sp. BD413 pMR30 (de Vries et al. 2003). No DNA was detected in surface soil under the growing potato plants (Widmer et al. 1997; de Vries et al. 2003). The same result was obtained with sugar beets, and even with surface soil sampled after harvest (which included the decapitation of the beets), as long as the samples did not contain visible plant material (Meier and Wackernagel 2003a). In other studies the tobacco plant gene for ribulose bisphosphate carboxylase (*rbc9*) was targeted by PCR and was found in soil 1 year after growth of plants (Paget et al. 1998). A number of studies have traced DNA released or present in plant litter deposited in soil (Widmer et al. 1997; Paget et al. 1998; Gebhard and Smalla 1999). It became clear that, depending on the sensitivity of the PCR assays, recombinant DNA was detected in the extracts prepared from the plant litter soil samples after several weeks or even a year (see Sect. 6.5.5). Plant DNA found in the environment may be present in live or dead plant tissue material, in dispersed pollen (see Sect. 6.5.4), or as extracellular DNA.

6.2.3 Other Organisms

Fungi and certain yeasts constitute significant parts of the soil microbiota and are expected to contribute to nucleic acid load in soil. Genetically modified derivatives of *Hansenula polymorpha* and *Saccharomyces cerevisiae* which are normally not found in soil have survived only at about a 1% level after 2 weeks (Tebbe et al. 1995) and during death may have released

DNA from cells. Fecal deposits of soilborne animals may contribute to the presence of free nucleic acids in soil from two main sources: they can contain (1) DNA from the animal gut epithelial cells and (2) DNA from organismic food of the animal including that from prokaryotic and eukaryotic microorganisms. It was demonstrated with mice that from DNA experimentally administered as food a measurable fraction can withstand degradation in the gut and is released as fragments up to 1.7 kb with the feces (Schubbert et al. 1994, 1997). It is conceivable that part of the DNA present in food and feed is released into the gastrointestinal tract of any animal and that a portion of it will be deposited in the environment with the feces (for references, see Jonas et al. 2001; Van den Eede et al. 2004). Widespread and numerically important animal groups in soil include nematodes, insects, and worms, specific members of which have been studied for microbial horizontal gene transfer (Adamo and Gealt 1996; Hoffmann et al. 1998; Thimm et al. 2001). In general, DNA that is present in soil may encompass genetic material of organisms living in soil and released in situ plus DNA from organisms living in other habitats but transported into soil by a number of mechanisms including translocation of organic material by wind, water, and animal movement.

6.3 Presence of DNA in Soil

Quantitative determination of the dissolved free DNA separately from DNA associated with cells or particulate material is a relatively easy task in aquatic environmental samples (Paul et al. 1988, 1989). With appropriate combinations of suitable filtration and precipitation steps it was shown that high molecular weight DNA of concentrations between 0.2 and 25 μg/l was present in marine and freshwater habitats (for references, see Lorenz and Wackernagel 1994). The isolation of DNA from soil samples is much more difficult because of the presence of DNA in variable cellular systems and the often rather strong associations of cells and of free DNA with different soil constituents. Various DNA isolation protocols have been worked out by a number of groups (e.g. by Ogram et al. 1987; Holben et al. 1988; Steffan et al. 1988; Pillai et al. 1991; Selenska and Klingmüller 1991; Tsai and Olsen 1991; Romanowski et al. 1992; Porteous and Armstrong 1993; Recorbet et al. 1993; Smalla et al. 1993; Porteous et al. 1997; Kruske et al. 1998; Bürgmann et al. 2001; Roose-Amsaleg et al. 2001) and several overviews have been presented (Van Elsas and Trevors 1995; Miller et al. 1999; Tien et al. 1999; Robe et al. 2003). This topic will be discussed in depth in Chap. 3, and will be dealt with here only briefly. Most techniques were designed to extract total DNA, i.e. DNA from possibly all kinds of cells present in soil plus extracel-

lular DNA, because the preparations were further employed for microbial community analyses, abundance determinations, and highly sensitive rare cell discovery. Therefore, the protocols include generally relatively harsh steps suitable to break microbial and other cells and even spores. These steps include ultrasonic treatment, very strong mechanical grinding like in a bead mill, hot sodium dodecyl sulfate (SDS) treatment (65 or 70 °C), or application of a chaotropic agent like guanidine thiocyanate. The resulting primary DNA preparations are mostly contaminated with dissolved soil components like humic acids which interfere with enzymatic procedures including restriction analysis and PCR amplification (Tebbe and Vahjen 1993). Thus, most protocols contain additional purification steps based on different methods including cesium chloride density gradient centrifugation, ion-exchange chromatography, selective adsorption of phenolic humic contaminants (mostly to polyvinyl polypyrrolidone), preparative gel electrophoresis, or specific precipitation. More recently, Frostegård et al. (1999) have compared different DNA extraction methods on four soil types from six different sites. They concluded that extraction and purification steps have to be optimised for the soil and the type of the DNA analyses. In order to reduce contamination of DNA with dissolved soil constituents and to specifically obtain DNA from soil prokaryotes (e.g. for community analyses) in a number of procedures, first the microbial cells are separated from soil particles and purified before DNA extraction (Torsvik 1980; Holben et al. 1988; Steffan and Atlas 1988; for further references, see Robe et al. 2003).

The question of whether extracellular DNA is present in soil and sediment and if so how it can be recovered has been a specific experimental challenge (Reanney et al. 1982). Mild elution techniques with buffer have been developed to desorb DNA from soil particulate material without breaking cells. A slightly alkaline 0.12 M phosphate buffer (pH 8.0; Ogram et al. 1987) and an alkaline Tris-HCl buffer with 0.5 M sodium chloride (TN588, pH 8.8; Blum et al. 1997) were employed in a number of studies. Buffers with a higher pH value and containing EDTA may sometimes allow more DNA to be recovered at the expense of much more extensive contamination of the DNA with humic substances (Frostegård et al. 1999; Blum and Wackernagel, unpubl.). Also in the extraction of proteins from soil with nearly neutral and EDTA-containing buffers the coextraction of humic substances was observed (see Chap. 4). In a direct comparison between the Ogram and the TN588 buffers the latter recovered about 50–60% of adsorbed [^{3}H]-thymidine-labelled DNA from three different non-sterile soils in a single elution step, while the phosphate buffer recovered between 15–30% (Blum et al. 1997). The DNA recovered by TN588 was essentially free of humic substances and could be directly used for hybridisation and PCR amplification (Blum et al. 1997). Later, the elution with TN588 was em-

ployed to demonstrate the presence of extracellular transgenic plant DNA in agricultural field plots (Meier and Wackernagel 2003a). A few studies documented that extracellular DNA is present in sediments and soils. The amounts can vary from about 1 to 50 μg per g of soil, but are generally lower than the amounts of DNA present in the cells of the soil organisms (Reanney et al. 1982; Ogram et al. 1987; Frostegård et al. 1999). These results were recently supported by the finding that with the Ogram buffer (see above) amounts between 2.2 and 41 μg of extracellular DNA were recovered per g of forest soil which amounted to between 10.5 and 60% of total DNA present (Agnelli et al. 2004).

6.4 Distribution of Extracellular DNA in Soil

When high molecular weight DNA is released from living or decaying cells into soil, then the dissolved molecules have the option to adsorb to solid soil constituents or remain in the liquid phase. This will result in a specific distribution of DNA within the soil. Most of the observations discussed in this section were made with soil microcosms. These are small samples of mineral or natural soil material in which the fate of DNA can be studied in the laboratory.

6.4.1 The Binding of DNA to Soils

Adsorption of DNA to mineral soil materials has been addressed in a number of studies. This topic will be discussed in detail in Chap. 7, and will be only briefly summarised here. The initial adsorption studies employed purified mineral soil components in the presence of mono- and divalent cations. The minerals were acid-washed sand (Aardema et al. 1983; Lorenz and Wackernagel 1987; Ogram et al. 1988; Romanowski et al. 1991) or its different constituents like quartz, heavy minerals, and feld spar (Lorenz and Wackernagel 1987), as well as various purified clay materials including montmorillonite, illite, kaolinite, and bentonite (Greaves and Wilson 1969; Khanna and Stotzky 1992; Lorenz and Wackernagel 1992; Paget et al. 1992; Poly et al. 2000). The effects of divalent cations in cation bridging (Greaves and Wilson 1969; Lorenz and Wackernagel 1987; Franchi et al. 2003) and of the linear or circular structure of the DNA on binding (Romanowski et al. 1991; Poly et al. 2000; Pietramellara et al. 2001) have been addressed. Divalent cations like Mg^{2+} and Ca^{2+} stimulate binding about 100-fold stronger than monovalent cations like Na^{+}, K^{+}, and NH_4^{+} (Lorenz and Wackernagel

1987; Franchi et al. 2003) and linear DNA was somewhat more effective in binding than supercoiled covalently closed DNA molecules. Studies with non-purified mineral material from the environment (natural groundwater aquifer material) showed that the "dirty" material (i.e. mineral particles covered with inorganic and organic precipitates and metal oxides and hydroxides) adsorbed DNA in the presence of groundwater rapidly and in quantities reaching more than 10 μg per g of material (Romanowski et al. 1993a).

Using natural soils with different characteristics the adsorption isotherms with purified DNA indicated generally high adsorption capacities which depended on the mineral content (particularly on the amount and type of clay), soil solution composition, and organic C content (Ogram et al. 1988, 1994; Blum et al. 1997). The binding of DNA was rapid and reached equilibrium values within 1 h at 23 °C for brown earth and a loamy soil or 3 h for podzol soil (Blum et al. 1997). The adsorption of DNA to soils also depends on the size of the DNA fragments. Ogram et al. (1994) showed that small fragments (2.96 kbp) were adsorbed preferentially to large fragments (23 kbp) from a polydisperse DNA solution. Smaller DNA molecules also bound more effectively on clay materials (Pietramellara et al. 2001). The greater extent of short DNA adsorption in various soils (with the exception of sand) may have resulted from slower diffusion of large molecules and from their exclusion from small pores in soil with binding capacity (Ogram et al. 1994).

6.4.2
The Distribution of DNA to Liquid and Solid Soil Phases

In an experimental approach to trace kinetically the fate of extracellular DNA in soil, three different non-sterile soils were loaded with [^{3}H]-thymidine-labelled DNA molecules of unique size (41.6 kbp) in microcosms (Blum et al. 1997). The DNA remaining unadsorbed in the interstitial soil liquid was recovered by washing out with the corresponding soil solution, and the DNA adsorbed on soil particulate material was then eluted with TN588 buffer. Within the first hours after addition of DNA to soil a soil-specific fraction of the DNA adsorbed on the solids while the rest of the DNA (20–70%) remained in the interstitium. This distribution did not dramatically change during the further incubation for 5 days. It was remarkable that the DNA in the interstitium was broken down into acid-soluble fragments ($\leq$20 bp) rapidly with soil-specific kinetics, reaching total degradation within 24–72 h, while the adsorbed DNA retained a high molecular weight status (acid precipitable). Another experiment showed that the adsorbed DNA was slowly released into the liquid phase during

5 days (30–50%) and then was almost instantly converted into acid-soluble material. The DNA remaining associated with the solids also decreased in molecular size during this time from greater than 20 kbp down to 2 to less than 0.75 kbp by soil-specific kinetics as measured by Southern transfer hybridisation (Blum et al. 1997). These experiments show that (1) extracellular DNA will distribute in natural soil to the liquid and solid (by adsorption) soil phases in a soil-specific pattern and (2) the fraction remaining in the interstitium will be degraded rather rapidly, while surface adsorption will provide partial protection against degradation in the liquid/solid interphase (see Sect. 6.5). In the experiments by Blum et al. (1997) and Ogram et al. (1994) the soil particulate material was not further fractionated to identify the components with which the DNA was associated. In a microcosm consisting of sea sand and clay (Na-bentonite) the majority of adsorbed DNA (60%) was found in the clay fraction, although this fraction was only 0.6% of the total mineral material in the microcosm (Lorenz and Wackernagel 1992). This corresponds with the much higher DNA-binding capacity of clays compared to quartz sand (Lorenz and Wackernagel 1994).

6.5 Persistence of DNA in Soil

The ubiquitous presence of nucleolytic enzymes in marine habitats, freshwater, sediments, and soils would not suggest long persistence of extracellular DNA in the environment. By use of antibiotics preventing bacterial growth it was shown that the majority of the DNase activity in the solutions of different soils comes from prokaryotic cells during their growth (Blum et al. 1997), mostly released as extracellular enzymes or sometimes associated with the cell surface (Basse et al. 1994). The level of DNase activity in the soil solution depends on soil type and can vary considerably during the vegetation period (Blum 1997; Blum and Wackernagel, unpubl.). In aquatic and sediment habitats considerable concentrations of extracellular high molecular weight DNA were detected despite the presence of DNases (for references, see Lorenz and Wackernagel 1994). Other studies suggested that also in soil habitats free DNA can be present (Torsvik and Goksoyr 1978; Reanney et al. 1983; Agnelli et al. 2004). This can be explained by assuming that nucleic acids released from cells can escape degradation for certain periods.

6.5.1 Protection of Mineral-Associated DNA against DNases

Most of the studies reported in this and the following section employed microcosms with soil or sediment or mineral soil components. The initial

findings were that the polyanion DNA can adsorb on the silicate anions of mineral surfaces at pH values above 2 when sufficient cations are present to lower repulsive forces between the negatively charged molecules and mineral surfaces and that the adsorption of DNA provides partial protection against its nucleolytic degradation (Greaves and Wilson 1970; Lorenz et al. 1981; Ivarson et al. 1982). The quantitative studies on adsorption and protection employing quartz sand, purified DNA, and DNase I (Lorenz and Wackernagel 1987) together with the important finding that the mineral-adsorbed DNA is still highly available for natural transformation of *B. subtilis* cells even in the presence of DNase (Lorenz et al. 1988) triggered extensive further studies (these are also discussed in Chap. 7). These studies employed sand and other soil mineral components including various clay minerals (Romanowski et al. 1991; Khanna and Stotzky 1992; Lorenz and Wackernagel 1992; Paget et al. 1992; Chamier et al. 1993) and samples taken from the environment such as groundwater aquifer material (Chamier et al. 1993; Romanowski et al. 1993a). DNA adsorbed on groundwater minerals in the presence of groundwater required a 1000-fold higher concentration of DNase I to be degraded to the same level as DNA dissolved in groundwater (Romanowski et al. 1993a). While the various protection experiments all involved DNase I (from bovine pancreas) as a model DNase, one study demonstrated protection of DNA bound on groundwater aquifer minerals also against a typical bacterial extracellular DNase, the highly active nuclease secreted by *Serratia marcescens* (Ahrenholtz et al. 1994).

The mechanism of protection was discussed as resulting from reduced access of DNase to the bound DNA and the sequestration of the DNase by its binding to the mineral surface (Lorenz and Wackernagel 1987; Khanna and Stotzky 1992; Paget et al. 1992). DNase I binds to Na-bentonite and montmorillonite and then its activity on added DNA is low (Khanna and Stotzky 1992). There is less binding of DNase I to quartz sand (Lorenz and Wackernagel 1987). Plasmid DNA recovered from sand loaded with supercoiled molecules and treated with DNase I consisted mostly of open circular and linear molecules suggesting that DNase I was active perhaps even after binding to the minerals (Romanowski et al. 1991, 1993a). In a recent study the protection of DNA bound on clay minerals against DNase I was mainly attributed to adsorption of the enzyme to clay (Demanèche et al. 2001). In these studies, however, DNase I was applied in H_2O in the absence of any divalent cations which are required for its activity ($\geq$ 5 mM Mg^{2+} plus $\geq$1 mM Ca^{2+}; Bollum 1965). Therefore, the results cannot be compared directly to previous studies in which sufficient divalent cations were provided for high DNase I activity in the assays to measure protection.

6.5.2 Degradation Kinetics of Introduced DNA in Soil

Two different approaches were used: introduction of purified DNA into soil and introduction of cells or tissue material containing the DNA to be traced. Introduced recombinant plasmid DNA was detectable in different non-sterile soils for up to 60 days (end of experiment) by dot blot and Southern hybridisation and transformation of $CaCl_2$-treated *E. coli* (Romanowski et al. 1992) and by quantitative PCR and electroporation of *E. coli* (Romanowski et al. 1993b). As an example, in loamy sand soil after 60 days, 0.2% of the introduced PCR targets (1029 bp of the *nahA* gene) were detected and 0.01% of the transforming activity which measured intact plasmids (Romanowski et al. 1993b). The degradation of the [^{3}H]-thymidine-labelled DNA resulted in acid-soluble material (Romanowski et al. 1992). Loss of PCR targets and transforming activity followed typical biphasic kinetics in the different soils. During the first 5 days the half-life times of the transforming potential of the plasmid DNA were relatively short (11.2–18.5 h) in the different soils and thereafter increased strongly (e.g. to about 28 days in loamy sand; Romanowski et al. 1993b). As it seems, a small part of the DNA had entered locations in soil in which degradation was slow. Widmer et al. (1996) also observed similar biphasic kinetics with linearised plasmid DNA introduced into a soil classified as silt loam, and stability was higher at 4 °C compared to 20 or 36 °C and at low soil moisture content (10% compared to 40 or 80%). After 40 days in soil up to 0.08% of the initial PCR targets were detected.

The introduction of *E. coli* cells genetically tagged for identification of their DNA by PCR amplification also revealed biphasic DNA degradation curves (see Sect. 6.3, Fig. 6.1; Recorbet et al. 1993) with a switch from rapid to slow DNA degradation rates after a few days in soil. Recorbet et al. (1993) found that the amount of extracellular DNA was only about 0.01% compared to the DNA still present in dead cells in soil after 40 days. Genomic DNA in transgenic plant material introduced into soil was also degraded rapidly during the first days, but remained detectable at a level of up to 0.14% after 120 days (Widmer et al. 1997). These results suggest that, similar to what was seen with naked DNA, also bacterial and plant tissue cells can find positions in soil in which their cellular DNA is rather refractory to degradation.

Generally, DNA in natural soil can be assumed to be present at least in four different settings which are (1) intracellularly in active cells, (2) intracellularly in dormant or dead cells, (3) associated with cell debris, or (4) as naked extracellular molecules. Both, the extracellular and the cell-associated states can occur in the soil solution or bound on or entrapped

in soil colloids. Similar locations in soil have also been discussed for active enzymes (see Chaps. 4 and 12).

The specific degradation kinetics of the DNA in each of the described locations are not known in detail because the DNA fractions present in these locations cannot easily be differentiated experimentally. At present the only approach to follow the degradation of extracellular DNA in soil solution and associated with soil particulate material separately but simultaneously was employing microcosms with non-sterile soil containing introduced radiolabelled DNA (Blum et al. 1997; see Sect. 6.4.2).

6.5.3 Methods to Assay the Persistence of Functional DNA in Soils

The most sensitive method to detect specific DNA fragments in soil is their amplification by PCR. The amplified sequences are mostly only parts of genes, often down to sizes of about 100 nucleotides (Recorbet et al. 1993) which does not tell whether the monitoring follows the persistence of large DNA molecules or smaller degradation products. More recently, a number of biomonitoring techniques have been presented which allow numbers of DNA molecules to be quantified by natural genetic transformation of competent bacteria (de Vries and Wackernagel 1998; Gebhard and Smalla 1998). This is an extension of earlier artificial transformation tests for plasmid DNA recovered from soil (Romanowski et al. 1992, 1993b; Tebbe and Vahjen 1993). Transformation tests do not depend on prior DNA amplification and therefore each transformant obtained represents one DNA molecule. Moreover, the natural transformation process is also largely insensitive to soil substances in DNA like humic acids which inhibit PCR (see Sect. 6.3). In fact, unpurified suspensions of soil or rhizosphere material can be directly added to the competent bacterial culture for transformation (de Vries et al. 2003). The sensitivity of these tests is similar to routine PCR (de Vries et al. 2003; Meier and Wackernagel 2003a) which requires about 1–10 ng of eukaryotic DNA to amplify a single copy gene (PCR Applications Manual, 2nd edn., Roche Diagnostics GmbH, Mannheim, Germany). The biomonitoring systems measure functional high molecular weight DNA, because DNA molecules are efficient in transformation only when larger than about 1 kb (for *Acinetobacter*: Palmen et al. 1993; for *P. stutzeri*: Meier and Wackernagel 2003a) and when their physical and chemical integrity allows homologous recombination in the recipient plus transcription and translation for expression of the inherited trait to take place.

One type of biomonitoring depends on marker rescue. In two cases the *nptII* gene (kanamycin resistance) was chosen which is present in

many genetically engineered crop plants (de Vries and Wackernagel 1998; Gebhard and Smalla 1998). The competent cells contained an *nptII* gene with an internal deletion which was filled up by recombination when the cells took up a DNA molecule with an intact *nptII* gene. These events were detected as kanamycin-resistant transformants. This system was used with *P. stutzeri* cells to monitor the spread of transgenic sugar beet DNA in field plots (Meier and Wackernagel 2003a) and with *Acinetobacter* cells (de Vries and Wackernagel 1998) to measure DNA release from plant root and tissue material (Tepfer et al. 2003). Integration of the *nptII* gene in recipient cells was absolutely dependent on the presence of nucleotide sequence homology between the transforming DNA and the recipient cell genome (de Vries et al. 2001). Recently, a similar biomonitoring using the *aadA* gene (spectinomycin resistance; integrated into the tobacco plastid DNA) with homologous flanking sequences was applied to follow the degradation kinetics of plant plastid DNA during decay of tobacco leaves (Ceccherini et al. 2003).

While the previous type of biomonitoring was specific for a particular gene (e.g. *nptII* or *aadA*), a more recently developed type of assay uses a genetic construct in the recipient cells which is specific for the recombinant DNA in a transgenic plant line, such as the recombinant fusion of a marker gene to the neighbouring nucleotide sequence (de Vries et al. 2003; de Vries and Wackernagel 2005). In these cases one crossover must occur in the marker gene (e.g. *nptII*) and the second in the adjacent DNA segment to fill up a deletion of the junction between the two sequences in the recipient in order to be scored as a kanamycin-resistant transformant. For example, this monitoring type can discriminate between DNA from transgenic plants all carrying the *nptII* gene but in different contexts. Such a system was applied in studies on the persistence of functional transgenic T4 lysozyme potato plant DNA in field releases (de Vries et al. 2003).

6.5.4 Persistence and Spread of Plant DNA in Agricultural Field Plots

Owing to the specific genetic tags of transgenic plants it has become possible to follow the fate of DNA from agricultural plants in field release experiments. In the study by Widmer et al. (1997) recombinant tobacco DNA could be retrieved from plant leaf litter in the field soil after 77 days at about 0.06% (detection limit) of the initial concentration (determined by quantitative PCR). Potato DNA persisted for 137 days at 2% (leaves and stem) and 0.19% (tuber) of the level of the plant material deposited in surface soil (Widmer et al. 1997). Paget et al. (1998) detected transgenic tobacco DNA by PCR of a 692 bp fragment in a sandy clay-loam soil up to

1 year after planting out. The authors assumed that part of the DNA might have persisted extracellularly. In a field with silt loam soil into which shredded material of transgenic sugar beets was introduced, recombinant DNA was detected by PCR in total DNA extracts after 2 years (650 bp fragment; Gebhard and Smalla 1999).

In field releases of transgenic sugar beets recombinant DNA was not only detected (by PCR in total DNA extracts) after planting out and growth of the sugar beets, but also in many (control) samples taken from the field 3 weeks before planting out of the sugar beets (in 13 out of 30 plots; Blum et al. 1997). This was surprising because never before had transgenic sugar beets been grown in these fields. In two of the 30 plots even extracellular transgenic DNA was recovered by the TN588 elution technique (Blum et al. 1997). Further analyses from other transgenic sugar beet field releases indicated that recombinant DNA was detected in surface soil from field plots or their vicinity only when sugar beet plants had been allowed to flower. This finding suggests pollen dispersal as the mechanism of DNA spread. Transgenic DNA was recovered from all 12 samples taken from a site for seed production or close to it and in few cases from samples taken 50 m away from the field (one out of ten) despite the fact that the pollen-producing plants were surrounded by a strip of hemp plants for pollen containment (Meier and Wackernagel 2003a). The transgenic DNA in the surface soil was detected by both PCR amplification and a bioassay (see Sect. 6.5.3). These observations explain why transgenic DNA was found in soil of a field with no previous growth of sugar beets: in the preceding year pollen from other fields with blooming transgenic plants had contaminated the site and the DNA had persisted till the next year when the sampling occurred. Similar observations were made with transgenic potato plants (de Vries et al. 2003). On the one hand, DNA was also released from the potato plant roots into the rhizosphere increasingly during growth from juvenile to senescent stages (de Vries et al. 2003). On the other hand, transgenic potato DNA was only detected in surface soil close to the plants and up to 2 m away (by PCR and a bioassay) when flowering of plants was allowed (de Vries et al. 2003). Altogether, these findings show that growing plants release their DNA by tissue material, pollen, and as extracellular DNA into the environment.

6.5.5
Long-Term Field Persistence of Plant DNA in Cellular Material or as Free DNA

The plant DNA isolated from field soil may come from plant cellular material that remained in soil or from free DNA, since the extraction procedures

mostly include treatments which destroy cells and would also detach DNA adsorbed on minerals. A recent study has focused on the decay of the transgenic tobacco DNA in leaf material during maceration following treatment with cellulase or bacterial pectinase and during wilting of leaves resulting from experimental infection with *Ralstonia solanacearum* (Ceccherini et al. 2003). The integrity of DNA measured by PCR and a bioassay dropped by about one to three orders of magnitude within 3 days. Interestingly, transforming DNA at about 2% of the initial activity was recovered from the wilting leaves after 8 days (Ceccherini et al. 2003). It appears that endogenous DNases in the destroyed tissue material are a main cause of DNA degradation. In this context it is interesting to note that evidence is available for relatively high DNA stability in pollen. It was observed that almost 1 year after growth and harvest of transgenic sugar beets recombinant DNA could be recovered from the field plots only when the plants had been flowering (Meier and Wackernagel 2003a). Moreover, samples of field soil probably contaminated with pollen of transgenic potatoes taken 1 year after potato harvest and stored moist for another 4 years still contained DNA which after extraction was detected by a bioassay (de Vries et al. 2003). Systematic studies on the long-term persistence of DNA in plant material decaying under natural field conditions and the release of DNA from this material into soil and its persistence in situ have not yet been performed.

6.6 The Extracellular Gene Pool Hypothesis

The continual release of DNA from organisms and tissue material in soil and its subsequent degradation with a time lapse will provide a pool of extracellular DNA consisting of DNA from all kingdoms of life with DNA from microbial sources probably being the major fraction due to their high abundance (as has been hypothesised for proteins in soil; see Chaps. 4 and 12). It has been proposed that this gene pool is mainly present on the surface of soil particles due to the higher DNase resistance which adsorption imparts to the DNA (Lorenz 1992). At present, cells of more than 80 prokaryotic species are known to have the potential for natural DNA uptake and many of them live in soil, sediment, and aquatic habitats (Lorenz and Wackernagel 1994; Tønjum et al. 1995; de Vries and Wackernagel 2005). The DNA may be used by the recipient cells as food or genetic material depending on sequence similarity and other factors (Redfield 2001; see also Chap. 15). Several soil bacteria were shown to take up DNA directly from the surface of mineral particles (*B. subtilis*: Lorenz et al. 1988; *P. stutzeri*: Lorenz et al. 1991; *Acinetobacter* sp. BD413: Chamier et al. 1993). The spontaneous stepwise desorption of DNA from solid surfaces during bacterial uptake

could result from the dynamic equilibrium between adsorbed (trains) and non-adsorbed segments of DNA (loops) on the mineral particles. Such a type of association is proposed by the model of Hesselink (1983) on the adsorption of polyelectrolytes on charged surfaces (Wackernagel 1996).

Acquisition of new genetic information by horizontal gene transfer including natural transformation (for a more detailed discussion of the situation in soil, see Chap. 15) is an important mechanism to generate diversity and thereby to promote the genetic adaptation and evolution of prokaryotes (Arber 2000). The extracellular gene pool in soil would provide access of transformable bacteria to a wide variety of genetic information including that of bacteria and plants. Once foreign DNA is taken up by a cell its genomic integration can occur by homologous (frequent) or homology-facilitated illegitimate (rare) recombination (de Vries and Wackernagel 2002; Prudhomme et al. 2002; Meier and Wackernagel 2003b). Recently, the integration of plant DNA into the genome of a soil bacterium during natural transformation and its expression was demonstrated, and the evolutionary implications of such events have been discussed (de Vries et al. 2004).

6.7 Conclusions

Soil is a habitat for millions of prokaryotes per g and is also inhabited by fungi, animals and plants. DNA is present in soil within the living and dead organisms and also in extracellular form after release during life and death phases of cells. Extracellular DNA distributes to soil liquid and to the surface of particulate material by adsorption in soil-specific kinetics and proportions. Depending on the soil, DNA in the liquid phase is degraded within hours or days by ubiquitous DNases which are mostly of prokaryotic origin. Mineral-adsorbed DNA is partially protected against enzymatic degradation. Despite continual degradation DNA can persist in soil for extended periods like months or even years. DNA of transgenic plants can be traced specifically by PCR amplification and other molecular and biological assays due to its unique recombinant constructs. Recombinant DNA has been detected in soil samples up to 5 years after growth of the transgenic plants. The DNA probably persisted within plant tissue material and pollen and perhaps even as free DNA. Typically, DNA introduced into soils either within cells or tissue material or as naked DNA was degraded by biphasic kinetics with rather rapid degradation at early times and much slower during later stages. DNA recovered even after 5 years had maintained its functionality in the sense that it could experimentally transform naturally competent bacteria including expression of its genetic information. It has been proposed that in soil the continual production and degradation of

DNA provides a dynamic extracellular gene pool of DNA from all soil organisms. The pool is mainly present on the mineral surfaces from which naturally transformable bacteria can take up genetic information.

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7 Stabilization of Extracellular DNA and Proteins by Transient Binding to Various Soil Components

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7.1 Introduction

Soils are chemically complex and spatially heterogeneous environments that offer a variety of DNA-adsorbing surfaces such as sands, silts, clays, and complex macromolecules such as humic acids and remnants of plant tissues. Different soil types vary in composition with respect to these surfaces, influencing the specific adsorption of DNA (Ladd et al. 1996). After adsorption to surfaces of quartz, feldspar, oxides or hydroxides containing heavy minerals, humic acids and clay, the DNA becomes partially resistant to degradation by extracellular nucleases (Aardema et al. 1983; Romanowski et al. 1991; Khanna and Stotzky 1992; Crecchio and Stotzky 1998). DNA fragments adsorbed on these surfaces have been shown to transform bacteria in vitro (Lorenz and Wackernagel 1990; Stewart et al. 1991; Khanna and Stotzky 1992; Paget et al. 1992; Romanowski et al. 1993; Pietramellara et al. 1997; Demanèche et al. 2001). The adsorption of DNA by soil particles is therefore important since it may extend the time period that extracellular DNA is available for natural transformation of bacterial communities (Gallori et al. 1994; Nielsen et al. 1997a,b). Table 7.1 summarises the results of studies that have examined the effects of soil particulate material and humic acids on the biological activity of DNA. These studies were carried out using purified DNA, DNA present in cell lysates from dead bacteria, or DNA naturally released from living donor bacteria (Khanna and Stotzky 1992; Gallori et al. 1994; Pietramellara et al. 1997; Nielsen et al. 2000a,b, 2004; Demanèche et al. 2001). Both prokaryotic and eukaryotic DNA have been used as sources of transforming DNA, as reviewed by Nielsen (2003) and Nielsen et al. (1998, 2001). Here, studies investigating the interaction of DNA (present in pure solution, cell lysates, and live donor cell suspensions) with soil components (present as pure substances, in mixtures, and as natural soil samples) are examined. The accessibility of adsorbed DNA

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Table 7.1. Microcosm studies on the availability of DNA adsorbed to humic acids, clay, and sand to competent bacteria

Conditions and sources of DNA	Humics/clays/ sands used	Recipient bacterium	Transferred genes and phenotypes	Reference
Humic acids				
Chromosomal DNA and plasmids of *Bacillus subtilis* bound to humic acids	Extracted from a forest soil	*B. subtilis*	*his* auxotrophy, chloramphenicol resistance	Crecchio and Stotzky 1998
Chromosomal DNA of *Acinetobacter* sp. incubated in a solution of humic acids	Commercial preparation	*Acinetobacter* sp.	*npt*II, kanamycin resistance	Nielsen et al. 2000a
Clays				
Chromosomal DNA of *B. subtilis* adsorbed to clay	Montmorillonite	*B. subtilis*	Various amino acid auxotrophs	Khanna and Stotzky 1992
Chromosomal and plasmid DNA bound to clay undergoing cycles of drying and wetting	Montmorillonite	*B. subtilis*	*trp* prototrophy, pHV14 chloramphenicol resistance	Pietramellara et al. 1997
Plasmid DNA bound on clays	Kaolinite, ilite, montmorillonite	*Acinetobacter* sp.	pGV1, pAVA213-8, kanamycin resistance	Demanéche et al. 2001
Sands				
Chromosomal DNA *of B. subtilis* attached to sand grains	Sea sand	*B. subtilis*	*trp* prototrophy	Lorenz et al. 1988
Chromosomal DNA of *Pseudomonas stutzeri* attached to sand grains	Sea sand	*P. stutzeri*	*his* auxotrophy	Lorenz and Wackernagel 1990
Plasmid and chromosomal DNA adsorbed on sand and groundwater aquifer material	Sea sand, groundwater aquifer material	*Acinetobacter* sp.	*trp* prototrophy, pKT210, chloramphenicol resistance	Chamier et al. 1993

molecules to soil microbes is discussed, with focus on their possible role as contributors to natural transformation processes in the open environment. In addition, the interaction of extracellular proteins (enzymes) with various soil components is briefly discussed.

7.2 DNA Interactions with Purified Soil Components

7.2.1 DNA Interactions with Sand

Several studies have shown that sand can bind DNA and that the adsorbed molecules are more resistant to degradation by nucleases than free DNA in solution. Ogram et al. (1988, 1994) determined that a direct linear relationship exists between the molecular weight of DNA and the degree of adsorption to a fine sand soil, where shorter DNA fragments adsorb better than longer fragments. The authors suggested that shorter DNA molecules may access a higher number of small pores within soil clay particles or that shorter molecules may be more rapidly exposed to a limited number of adsorptive sites in soil due to higher diffusion rates. Aardema et al. (1983) and Lorenz and Wackernagel (1987) investigated adsorption of DNA (120 μg) from *Bacillus subtilis* and calf thymus to quartz sand (0.7 g) in glass columns. The highest adsorption of DNA was reported after 2 h at 23 °C, increasing with salt concentration (0.1–4 M NaCl or 1 mM–0.2 M $MgCl_2$). The adsorbed DNA could transform *B. subtilis* cells attached to sand grains, and the overall transformation frequencies were 20- to 50-fold higher than those obtained in liquid culture (Lorenz et al. 1988). Bacterial transformations have also been reported using sand-attached *Pseudomonas stutzeri* cells (Lorenz and Wackernagel 1990) and *Acinetobacter* sp. cells exposed to plasmid DNA (Chamier et al. 1993).

7.2.2 DNA Interactions with Clay Minerals

Due to isomorphous substitutions and charged edges, clay minerals can efficiently bind DNA or other charged molecules by direct or indirect adsorption. The positively charged edges are responsible for the direct adsorption of DNA, whereas divalent (Ca^{2+} and Mg^{2+}) or trivalent (Al^{3+}) cations provide a neutralising bridge between negative charges on clay surfaces and DNA macromolecules (Greaves and Wilson 1969; Paget et al. 1992). Clay minerals are capable of adsorbing large quantities of DNA; for instance,

below pH 5, purified montmorillonite can adsorb more than 16 mg of DNA per mg of montmorillonite (Greaves and Wilson 1969). DNA adsorbed on clay minerals such as montmorillonite, illite, and kaolinite is protected against rapid degradation by nucleases in soil (Greaves and Wilson 1969, 1970).

Effects of DNA Structure and Composition on Binding to Clay Surfaces

Poly et al. (2000) reported a correlation between the type and distribution of charge density of a DNA molecule and its specific adsorption to clay minerals. Low molecular mass supercoiled plasmid DNA showed the strongest binding and the highest charge density. It can be hypothesised that tightly coiled DNA molecules more easily overcome physical hindrances by forming few but strong bonds on the edge of the clay minerals. In contrast, longer linear DNA molecules with lower charge density experience stronger physical hindrances and likely form several weak bonds on the planar surface of the clay particles. However, contrasting observations suggest that chromosomal DNA binds more strongly to clay surfaces than plasmid DNA and RNA (Khanna et al. 1998; Franchi et al. 1999). Moreover, low molecular weight DNA was found to adsorb better onto montmorillonite and kaolinite than did high molecular weight DNA (Pietramellara et al. 2001). Pietramellara et al. (2001) reported that adsorption of DNA to clay minerals is not affected by base composition or by the presence of blunt versus cohesive ends. However, the DNA's negative phosphate groups were important for adsorption on clay minerals by generating cationic bridges with the clay's charge-compensating cations or by direct binding to its positively charged edges (Lopez-Hernandez et al. 1986). Other factors including pH and the presence of organic anions are also important for binding to occur (Greaves and Wilson 1969; Lopez-Hernandez et al. 1986; Paget et al. 1992; Pietramellara et al. 2001).

Localisation of DNA Molecules on Clay Surfaces

Analyses of DNA–clay complexes by X-ray diffractometry and electron microscopy have shown that DNA binds primarily to the edges of clay surfaces and that the remaining DNA "tail" extends freely outwards (Paget and Simonet 1994; Khanna et al. 1998; Franchi et al. 1999). Paget and Simonet (1994) and Khanna et al. (1998) suggested that DNA was adsorbed on the clay's external planar surface through weak bonds, whereas stronger bonds were formed when DNA molecules interacted with the clay edges. Weaker binding sites were most prevalent on the surface of montmorillonite, whereas the stronger binding sites of the clay edges were predominant in kaolinite. Considering a Schatcard-plot analysis of DNA adsorption on montmorillonite and kaolinite, Pietramellara et al. (2001)

hypothesised that DNA interacted with a higher number of bonds on kaolinite than on montmorillonite. It was suggested that adsorption of nucleic acids mainly occurs on the edges of clay mineral surfaces and that kaolinite presents higher ratios of edge surface/planar surface, anionic exchange capacity/surface charge density, and anionic exchange capacity/cationic exchange capacity than montmorillonite (Stotzky 1986; Paget and Simonet 1994; Alvarez et al. 1998; Franchi et al. 1999; Pietramellara et al. 2001). Determination of electrophoretic mobility and particle size of plasmid DNA complexed with montmorillonite and kaolinite with a Doppler effect light scattering analyser (DELSA) showed that the mobility of the complexes increased with decreasing amounts of DNA bound on the clay minerals and with lower pH of the suspension. In addition, the size of the DNA–clay complexes expanded after increasing the amount of DNA bound to the clay minerals (Pietramellara, unpubl. results).

7.2.3 Natural Transformation of Bacteria with DNA Adsorbed or Bound to Clays

The availability of clay-bound DNA to competent bacteria has been addressed in several laboratory studies using purified clays. Khanna and Stotzky (1992) investigated the natural transformation of *B. subtilis* with chromosomal DNA adsorbed to montmorillonite. Both adsorbed and desorbed DNA transformed competent cells. Pietramellara et al. (1997) studied the effects of varying water content on the transforming ability of chromosomal and plasmid DNA bound to montmorillonite and kaolinite by using competent *B. subtilis* cells. Chromosomal DNA retained the ability to transform bacterial cells after 3–4 cycles of wetting and air-drying, whereas plasmid DNA lost its transforming activity after 1–2 cycles. However, 6 h of wetting were required for the clay–DNA complexes to regain transforming activity. The authors hypothesised that changes in the structural conformation of the DNA molecule occurred under drying conditions, making it unable to transform competent bacterial cells, possibly due to a B- to Z-DNA form transition (Guéron et al. 2000).

Alvarez et al. (1998) reported that DNA from *B. subtilis* and calf thymus bound to montmorillonite clays could be amplified by polymerase chain reaction (PCR), thus suggesting that no irreversible binding or chemical modifications take place that would hinder possible transformational activity of such DNA. A recent study by Demanèche et al. (2001), using in vitro models of plasmid DNA adsorbed to clay minerals (kaolinite, illite, montmorillonite), revealed that clay-bound DNA was only partially available for transformation of *Acinetobacter* sp. BD413. They suggested that the

amount of DNA available was correlated to the concentration of competent bacteria and the extent of nuclease degradation. For many competent bacteria, uptake of DNA is initiated by the fragmentation of DNA into smaller fragments by endonucleases where DNA cleavage is carried out on the bacterial surface before the uptake of DNA through the membrane(s) (Lorenz and Wackernagel 1994).

7.2.4 DNA Interactions with Humic Substances

Humic substances are common in soil. Up to 10% of the total organic phosphorus (P) in soil is associated with DNA and humic acids (Baker 1977). The heterogeneous nature of humic acids makes their specific interactions with DNA difficult to determine. The persistence and degradation of nucleic acids adsorbed onto clay and humic surfaces are commonly assessed by comparison with the stability of dissolved nucleic acids in solution. To investigate whether humic acids protect DNA against nuclease activity, Crecchio and Stotzky (1998) exposed chromosomal DNA and plasmids obtained from *B. subtilis* to humic acids extracted from a forest soil in vitro. Up to 50 μg of DNA were bound to 3 mg of humic acids at pH interval 3–4. The bound DNA had higher resistance to DNase I degradation than free DNA. DNA isolated from soil is often co-extracted with humic substances although it is often unclear if this is a result of the extraction procedure or due to close association between humic acids and DNA in soil (Saano et al. 1993; Tebbe and Vahjen 1993).

7.2.5 Natural Transformation in the Presence of Humic Substances

Crecchio and Stotzky (1998) exposed chromosomal DNA and plasmids obtained from *B. subtilis* to humic acids and found that the DNA retained the ability to transform competent recipient cells of *B. subtilis*. Similarly, Nielsen et al. (2000a) reported that the presence of humic acids was non-inhibitory to natural transformation of *Acinetobacter* sp. cells in vitro. Moreover, incubation of chromosomal DNA in a solution of humic acids over 7 days did not substantially reduce the number of bacterial transformants obtained, as compared to solutions with pure DNA, indicating that humic acids do not negatively affect the transforming activity of extracellular DNA.

7.3 Protein Interactions with Purified Soil Components

7.3.1 Protein Interactions with Clay Minerals

Most extracellular proteins in soils are associated with either clay or organic colloidal fractions, and the persistence and stability of proteins in soils can be attributed to their association with clays and humic substances (Boyd and Mortland 1990). Proteins, generally, are rapidly adsorbed on clays within the first hours of contact (Ding and Henrichs 2002). Adsorption is reversible to a limited extent and only minor amounts of protein can be brought into solution after washing of the clay–protein complexes. In these complexes, proteins that cannot be desorbed are often referred to as "bound protein" (Stotzky 1986). The maximum adsorption amounts depend on clay type, decreasing in the order: smectite > vermiculate = illite > kaolinite, and are generally related to clay properties such as surface area, cation-exchange capacity, charge density, type of saturating cation, and degree of clay swelling. Intercalation of protein in swelling clay minerals has been observed in the laboratory but has not been reported for low or trace amounts of protein in solution, which may explain why intercalated protein–clay complexes have not been found in natural soils (Stotzky 1986; Boyd and Mortland 1990). Protein adsorption appears to be pH-dependent with maximal amounts adsorbed in the range of the protein's isoelectric point, suggesting that ion exchange is the main mechanism of adsorption (Stotzky 1986; Boyd and Mortland 1990). In addition to ion exchange, hydrogen bonding, van der Waal forces and hydrophobic effects have been shown to be important, depending on the properties of the clay surfaces (Quiquampoix et al. 2002).

Proteins can undergo conformational changes when adsorbed onto clay surfaces, depending on the protein and type of adsorbent surface (Fusi et al. 1989; Quiquampoix et al. 2002). Changes in protein conformation are reversible (Ding and Henrichs 2002). A general reduction, but not a complete elimination, of the catalytic activity of enzymes has been observed after clay adsorption, and higher K_m and lower V_{max} values have been reported for adsorbed enzymes in comparison with those in solution (Nannipieri and Gianfreda 1998). This indicates a loss of specificity and activity that could result from either a modification of the tertiary structure of the enzyme, and therefore its active site, or from reduced accessibility to the active site for the substrate. Other proteins (e.g. the larvicidal toxins of *B. thuringiensis*) retain their biological activity after adsorption onto clays as a function of the clay loading and gain longer resistance to microbial degradation (Stotzky 2002; Lee et al. 2003). The biodegradation of proteins

complexed with smectite has also been shown to decrease in comparison with free protein (Calamai et al. 2000; Lozzi et al. 2001).

7.3.2 Protein Interactions with Humic Substances

Proteins may exist in association with humic substances in soil (Nannipieri et al. 1996). The interactions and enzymatic activities of protein–humic substance complexes have been studied in the laboratory using three experimental approaches (Nannipieri et al. 1996; Ruggiero et al. 1996): (1) natural complexes extracted from soils, (2) model complexes prepared with extracted humic substances and enzymes, and (3) model complexes with enzymes and humic-like substances. The results of the different approaches show that extracellular enzymes in soil are firmly associated with humic organic matter and that, in the complexed state, enzymatic activity and bioavailability decreased, or in some cases was eliminated (Nannipieri et al. 1996). Several physical-chemical mechanisms involved in humus–enzyme interactions were hypothesised: (1) covalent and hydrogen bonding (Rowell et al. 1973); (2) ionic and hydrogen bonding (Sarkar 1986); (3) trapping into the macromolecular net by physical forces (Maignan 1982; Serban and Nissenbaum 1986); and (4) formation of an electron donor–acceptor complex (Gosewinkel and Broadbent 1986). The precise location of proteins in soil is important for their stability as proteins adsorbed to the humic matrix are vulnerable to proteolysis whereas proteins entrapped by the humic matrix may become resistant to proteases (see Nannipieri et al. 1996).

7.4 Interactions of DNA, Combined with Other Cellular Substances, with Pure Soil Components

Studies on the adsorption and binding of DNA on soil colloids (clay minerals and humic substances) and sand particles are mostly carried out with pure DNA (Khanna and Stotzky 1992; Gallori et al. 1994; Ogram et al. 1994; Paget and Simonet 1994; Pietramellara et al. 1997; Demanèche et al. 2001). However, these studies do not reflect in situ conditions where nucleic acids are present and released in heterogeneous suspensions of cell debris (Nielsen et al. 2000a; Nielsen and Van Elsas 2001; Ray and Nielsen 2005). Bacterial cell death and lysis produce suspensions consisting of cell membranes, DNA and remaining cytoplasmic content (Lorenz et al. 1991). These conditions and the presence of different microenvironments in soil

must be considered when investigating the persistence, degradation and bioavailability of extracellular DNA molecules.

7.4.1 DNA–Protein Interactions

Proteins associated to DNA molecules can bind to each other in a long cluster along the DNA molecule, generating a cooperative effect between adjacently bound proteins (oligomerisation capacity; Griffith et al. 1994). Proteins interacting with DNA molecules are more resistant to protease degradation. Due to the protein interactions, DNA can gain higher protection against nucleases (Setlow et al. 1995). The protective effect of protein binding on DNA stability is exemplified through established molecular techniques such as gel-shift assays where the location of DNA-binding proteins is determined through their protective effects during nuclease treatment of the DNA–protein mixture. Setlow and Setlow (1993) and Setlow et al. (1995) showed that small acid-soluble proteins that coat the DNA of *B. subtilis* spores increase the resistance of DNA to hydrogen peroxide and heat. Thus, the various DNA-binding proteins not only affect the adsorption of extracellular DNA on soil particles such as clay particles (see below), but also influence the resistance of extracellular DNA to environmental stress and enzymatic degradation (Haynes et al. 2000; Nielsen et al. 2000a). Nevertheless, we are not aware of in situ studies (e.g. in soil, water or digestive systems) that have examined the possible role of DNA-binding proteins in enhancing the stability of extracellular DNA molecules and their effect on the uptake of DNA into competent bacteria.

7.4.2 Adsorption of DNA–Protein Complexes on Different Soil Components

Proteins are rapidly adsorbed (< 1.5 h) on clay minerals through electrostatic interactions (Ding and Henrichs 2002). Proteins coating DNA and clays may therefore affect DNA binding to soil substances (Tavares and Sellstedt 2001). The adsorption of DNA onto surfaces is also the first step of biofilm formation (Donlan 2002; Whitchurch et al. 2002). Ascher et al. (2002) compared the adsorption of purified DNA or DNA in lysates containing coating proteins onto Ca^{2+}-saturated montmorillonite and kaolinite. The study showed that proteins coating the DNA enhanced the amount of DNA adsorption to clay minerals. However, the binding of protein–DNA complexes on clay minerals did not increase resistance against degradation by nucleases.

7.5 DNA Interactions with Natural Soils

Soil microorganisms degrade all natural organic compounds including DNA (Blum et al. 1997; Nannipieri and Badalucco 2002). The decay rate of DNA in soil is affected by microbial activity, pH, temperature, faunal predation, extracellular nuclease activity, soil components and structure, valence and concentration of cations, water transport and the molecular size and characteristics of DNA, as well as the integrity and composition of dead cells. The persistence of extracellular DNA in the soil environment can range from a few days to several years, as determined by the biological, physical and chemical characteristics of the DNA donor and soil matrix. Studies on the persistence of DNA in soil most often only considered the top profile (0–15 cm) of the soil where the content of organic matter and microbial and extracellular enzyme activities are higher. The amount of DNA in soil has been reported to decrease along the soil profile (Fritze et al. 2000; Agnelli et al. 2004) although DNA can be transported with water flow over considerable distances in agricultural soil, forest soil and groundwater (Poté et al. 2003; Agnelli et al. 2004).

DNA from eukaryotic cells differs from that from prokaryotic cells in structural complexity, cellular arrangement and molecular size; such differences may be of importance for DNA adsorption and degradation rates in natural environments. Moreover, the conditions causing cell death must be considered when examining the release, persistence and structure of DNA molecules in natural environments (Ceccherini et al. 2003). These conditions include (1) disruption of cells by physical damage to the organism, (2) spontaneous or pathogen-induced cell lysis, (3) necrosis in eukaryotic cells, (4) apoptosis, genetically programmed cell death, where the genetic material is partly destructed in the process by endonucleases, and (5) terminal differentiation of cells in which cells lose their nuclei but remain functional. The relative effect of these cellular processes on the persistence of DNA from various organisms is poorly understood, and difficult to determine in detail for different species in soil.

Many substances have been identified in soil, such as humic substances, sand, and clay minerals, which adsorb and protect DNA molecules from rapid degradation by nucleases. In natural soils, the adsorbing components are mixed together with various types of oxides and hydroxides to form "aggregates" that are colonised by microorganisms. The presence of extracellular DNA molecules inside soil microaggregates or micropores, which are inaccessible to microorganisms or extracellular nucleases, could explain the discrepancy between the observed long-term physical stability of DNA and the short-term biological accessibility of the DNA to

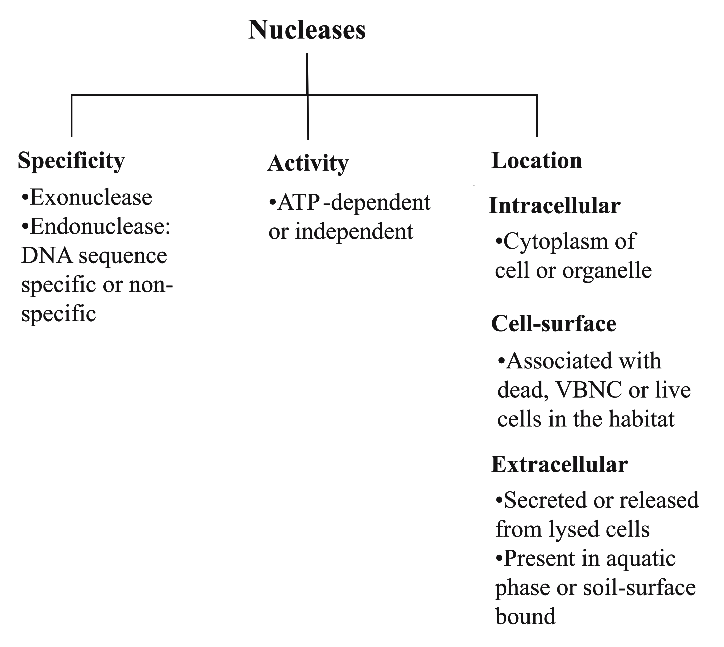


Fig. 7.1. A general overview of the substrate specificity, energy requirement and cellular location of DNA nucleases

soil bacteria (Blum et al. 1997; Benedik and Strych 1998; Nielsen 2003). Figure 7.1 outlines some characteristics of DNA nucleases active in soil environments.

7.6 Protein Interactions with Natural Soils

Protein–soil interactions have been extensively studied in laboratory experiments over the last 40 years. For reviews, see Skujins (1976), Theng (1979), Boyd and Mortland (1990), Gianfreda et al. (2002), Pietramellara et al. (2002), and Quiquampoix et al. (2002). As many as ten different classes of enzymes have been identified in soils (Ladd 1978; Ruggiero et al. 1996; Nannipieri et al. 2002; Tabatabai and Dick 2002). The most abundant classes of enzymes in soil detected by the present enzyme assays are hydrolases and oxidoreductases. Proteins, including enzymes released from organisms in soils, may undergo different fates depending on their location (Fig. 7.2). Once released from the cell, these proteins are rapidly used as a nutrient source by soil microorganisms, unless stabilisation occurs via adsorption and/or incorporation in clay and clay–humic complexes. Except for glo-

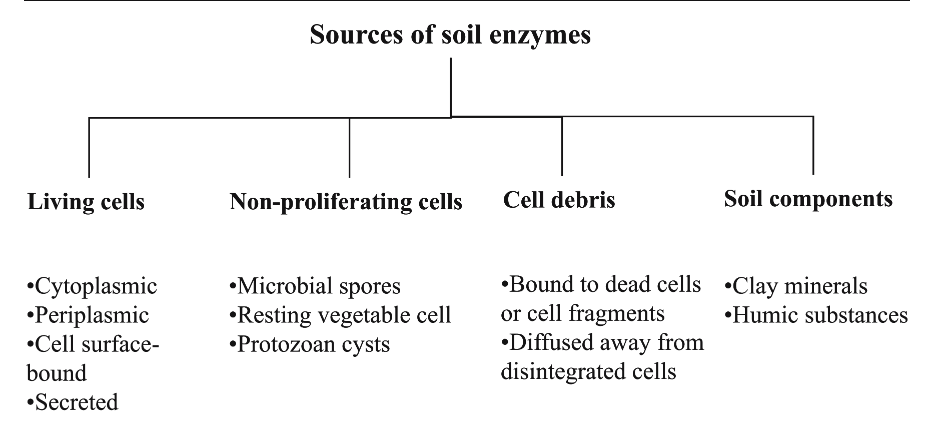


Fig. 7.2. A general overview of the sources and locations of enzymes active in soil

malin, an arbuscular mycorrhizal glycoprotein produced in large amounts by fungi in soil (Wright and Upadhaya 1996), and urease (Briggs and Segal 1963), the extraction and purification of specific proteins from soil is problematic (see Chap. 4). Hence, the presence, location, and concentration of specific proteins possessing enzymatic activity (such as nucleases) are often traced by enzymatic assays. Alternatively, immunoassay detection coupled with fluorescence or electron microscopy techniques may be used (Ladd et al. 1996). For instance, active acid phosphatase could be found in membrane fragments as small as 7×20 nm using immunocytochemical techniques (Ladd et al. 1996, and references cited within). However, the direct demonstration of extracellular enzyme activity on clays and other soil particles is problematic due to the presence of electron-dense material interfering with the immunocytochemical techniques used.

7.7 Concluding Remarks

Several studies have examined the factors affecting protein- and DNA-adsorbing capacities of soils. It has been found that soils with higher clay content adsorb more protein and nucleic acids than those with lower clay content (Ladd et al. 1996). Most studies on the protein or DNA adsorption capacities of soils have been performed in vitro using purified soil components such as clay or sand minerals. The in situ relevance of these studies is questionable because clay surfaces are expected to be covered by organic materials or inorganic components in natural environments. For instance, clay minerals can be coated by Fe-Al oxyhydroxides and humic substances in soil. Also, Lopez-Hernandez et al. (1986) reported that organic anions

such as those found in root exudates (malate and oxalate) reduce phosphate adsorption by soil. In the rhizosphere, these organic anions may therefore reduce the binding of extracellular DNA or proteins. The actual availability of DNA- and protein-adsorbing surfaces provided in soils under natural conditions should be further clarified and advances in technique development are necessary to dissect the in situ molecular interactions of DNA and proteins with specific soil components such as clays. It is expected that clay- or sand-bound DNA or proteins that are available to bacteria will also, to some extent, be physically accessible to a variety of extracellular or cell-surface-bound microbial nucleases or proteases. To our knowledge, it has not been demonstrated that nucleases of DNA-degrading bacteria and nucleases produced by competent bacteria differ in their ability to access extracellular DNA. Limitations to the study of the fate of extracellular DNA in soil are caused by the extreme heterogeneity of the soil environment that complicates in situ studies as well as limits the sensitivity and specificity of DNA extraction and purification techniques in soil. Similar constraints apply to understanding the fate of proteins in soil. Nevertheless, there has been a recent increase in efforts to develop sensitive methodology for the in situ localisation and quantification of extracellular DNA and proteins in various complex environments such as soil. This renewed interest is partly generated from investigations monitoring the environmental fate of recombinant DNA and proteins released from genetically modified organisms.

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8 Assessing Bacterial and Fungal Community Structure in Soil Using Ribosomal RNA and Other Structural Gene Markers

George A. Kowalchuk, Barbara Drigo, Etienne Yergeau, Johannes A. van Veen

8.1 Introduction

Soil-borne microbial communities are by far the greatest source of biodiversity on Earth, and recent use of molecular biological techniques has given us new tools to discover and monitor complex microbial communities in soil (Pace 1997; Torsvik et al. 1998, 2002). Most widely studied by these molecular approaches have been the Bacteria, but similar methods have become available to examine Archaea and Eukaryotes, especially fungi. The vast majority of methods to address microbial community structure to date have relied on polymerase chain reaction (PCR)-based technologies that target ribosomal RNA (rRNA) genes, principally the small subunit rRNA gene (SSU rRNA). Although this marker has proven invaluable in discovering novel microbial diversity (Hugenholtz et al. 1998), reliance on this single gene clearly presents an incomplete picture, and a number of other genetic markers have recently been introduced into the study of microbial community structure (Ludwig and Schleifer 1999). The latter include general markers coding for conserved proteins involved in core cell functions, or genes that are essential to activities defining specific functional groups, such as ammonia oxidation, nitrogen fixation, nodulation, or xenobiotic degradation. To provide a complete review of nucleic-acid-based microbial detection systems would be impossible within the confines of this chapter. Rather, in this chapter, we seek to provide a critical examination of the choices and alternatives available for the analysis of soil-borne microbial communities and discuss recent and future trends in the field. In doing so we will highlight the advantages and limitations of various methods, emphasising the importance of tailoring one's choice of approach and methodology to the research question and study subject. As with most molecular biological methods, no single method is a panacea. By and large,

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a combination of approaches will provide more reliable information than any single approach.

8.2 The General Choices in Molecular Analysis of Soil-Borne Microbial Communities

The difficulties associated with the *in situ* identification, isolation and cultivation of microorganisms from soil have provided the impetus to develop molecular biological methods for the analysis of soil-borne microbial communities. In addition, the vast complexity of soil systems impedes steps to understand the structure and functioning of communities in this habitat. Indeed, even with the most elaborate and exhaustive molecular methods, the vast diversity of organisms inhabiting soil (Torsvik et al. 1990) precludes our ability to characterise all the organisms in this habitat completely. Clearly, choices have to be made as to the scope of the organisms to be examined and the detail to which they can be described (Fig. 8.1). There is generally no single 'correct' route to follow, as each approach has

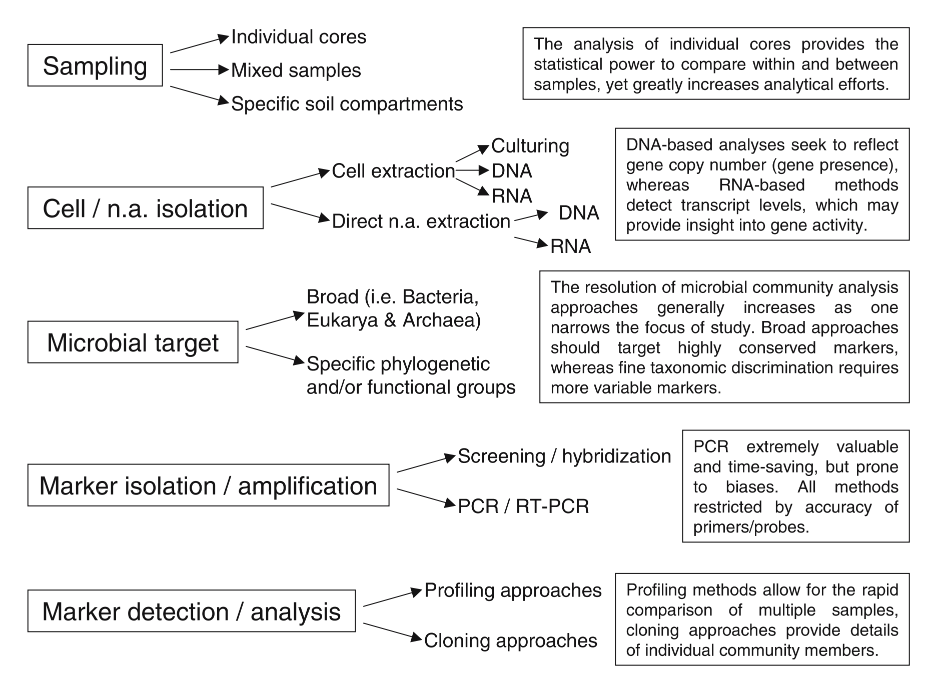


Fig. 8.1. Major considerations when choosing the microbial community analysis approach

its inherent limitations. The goal is to fit one's choice of approach to the research question and the type of information that is desired.

Any individual experimental design must provide maximum data output, while remaining feasible within budgetary and personnel limits. There has been a general dichotomy between in-depth studies, which lack statistical power because resources limit their scope to very few samples, and multiple sample comparisons, which often remain superficial. The introduction of new group-specific profiling methods, more powerful sequencing capabilities and microarray technologies (see also Chap. 9) may, however, help to break down this dichotomy in the coming years.

8.2.1 Community Structure Versus Diversity

Microbial diversity is a highly misused term. Many studies using molecular approaches to describe microbial communities mistakenly refer to assessments of microbial diversity, when a more accurate description might be "tentative identification of some of the most abundant community members". Of course, the term 'microbial diversity' sounds much better than such cumbersome statements in research articles and grant applications, but it is usually incorrect. Terms such as "community structure" or "structure of dominant populations", or the like, are generally more accurate. Indeed, given the enormous diversity contained within soil-borne microbial communities, we are typically restricted to analysis of some of the more dominant members, i.e. those that we sequence in clone libraries or see as bands in profiling experiments. All measures of diversity involve determinations of the number of species present, and as this is rarely determined in assessments of soil-borne microbial communities, precluding the ability to make diversity assessments.

This is not to say that some approaches cannot yield information pertinent to estimates and calculations of diversity. In particular, cloning surveys can provide molecular data equivalent to the collector's curves obtained by macro-ecologists when sampling species (Curtis et al. 2002). However, in broad analyses (e.g. at the domain level), the level of sampling (i.e. number of clones examined) still falls far short of that necessary to provide a full coverage of the species present, even in the most extensive studies performed to date (Borneman et al. 1996; Borneman and Triplett 1997; McCaig et al. 1999; Dunbar et al. 2002; Zhou et al. 2002, 2004). This is complicated by the fact that we often lack comprehension of what entails a microbial species or an ecologically significant microbial unit (Hughes et al. 2001). This problem has typically been circumvented by defining diversity in terms of arbitrary units consisting of members that share a preset

level of similarity within the chosen marker sequence. Such operational taxonomic units (OTUs) make no reference to functional or reproductive continuity. Although molecular surveys to date fall short of providing a full description of microbial diversity, application of ecological theory to available datasets does provide important insight into microbial diversity and allows comparison across samples (Hughes et al. 2001; Curtis et al. 2002; Dunbar et al. 2002). However, the right-end tail of the distribution curve (i.e. the rarest species in soil samples) has yet to be evaluated, and it is still not known if microbial communities share the same species distribution patterns as observed for macroorganisms. Further developments are necessary for the analysis of larger datasets and comparisons of higher order structure within microbial molecular inventories (Schleifer 2004). High-throughput sequencing efforts may allow us to venture into the right-end tails of species distribution curves, even for highly diverse soil communities, and rapid ribotype screening methods may simplify this task (Valinsky et al. 2002a,b; Neufeld et al. 2004). Analysis of the full distribution of species becomes a far less daunting task if one decides to focus on a specific phylogenetic or functional group. Interestingly, available evidence suggests that different subsets of microbial communities may have very different species diversity patterns (Kowalchuk et al. 2002; Horner-Devine et al. 2003) and spatial heterogeneity at microbially relevant scales may be important in shaping microbial communities (Yin et al. 2000; Zhou et al. 2002, 2004).

Microbial diversity estimates based on the number or the number and intensity of signals derived from microbial community profiling methods (e.g. DGGE, TGGE, T-RFLP, SSCP, etc.) are generally flawed. To begin with, detection is typically limited to only the most dominant community members (> 1%, see below; Muyzer and Smalla 1998; Brüggemann et al. 2000; Engebretson and Moyer 2003). This focus primarily on more dominant microbial populations is not unique to the resolution of community profiling methods, but is also true of other culture-dependent and -independent methods.

Even if one disregards rRNA gene copy number effects and potential PCR biases (see Sect. 8.2.2), band number and intensity cannot be accurately translated into species numbers and abundance. Problems with such diversity extrapolations stem from co-migration of sequences from different species (Sekiguchi et al. 2001) and multiple banding patterns derived from a single species due to imperfect running behaviour (Clapp 1999; Schmalenberger et al. 2001), primer ambiguities (Kowalchuk et al. 1997a), or intraspecific sequence heterogeneity (Clayton et al. 1995; Nübel et al. 1996; de Souza et al. 2004).

Obviously, it is impossible to represent thousands of species as a banding pattern within a limited amount of space on a gel, and a large disparity exists between the seemingly immeasurable diversity found via molecular

clone inventories of soil communities and the limited number of bands detected by community profiling methods. This disparity stems not only from the inherent focus on more dominant populations, but also false sequence variation introduced into clone libraries by PCR and cloning artifacts (Wang and Wang 1997; Speksnijder et al. 2001). As diversity of the target group or the sample fraction decreases (Holben et al. 2004), so will the limitations outlined above, but estimates of microbial diversity from community profiles remain risky.

8.2.2
The Benefits and Limitations of PCR-Based Approaches

The ability to amplify specific target sequences from complex nucleic acid mixtures with the aid of PCR has revolutionised microbial ecology. PCR technologies, with the aid of appropriate primers, have made it possible for researchers to investigate rapidly genes of interest in the environment without the need to culture the organisms that harbour these genes. However, with the utility of PCR, it is easy to forget that the first molecular microbial surveys were actually conducted without the benefit of PCR (Olsen et al. 1986; Pace et al. 1986). These groundbreaking studies relied on the painstaking screening of shotgun clones taken directly from environmental DNA. The ability to amplify specifically rRNA genes from environmental samples by PCR provided the breakthrough for rapid discovery of vast amounts of microbial diversity (Giovannoni et al. 1990; Hugenholtz et al. 1998). While this approach is unparalleled in power to uncover novel microbial diversity, it is not without its flaws, especially with respect to accurately representing true community structure. PCR amplifies target DNA molecules, but does not necessarily do this perfectly: PCR-induced biases include polymerase errors, preferential amplification of certain targets over others (Suzuki and Giovannoni 1996; Polz and Cavanaugh 1998), and formation of chimera and heteroduplex molecules (Kopczynski et al. 1994; Wang and Wang 1997; Speksnijder et al. 2001). Respectively, these problems can lead to sequence errors, unrepresentative recovery of target molecules, and false sequences. Although some of these issues can be minimised by the use of appropriate protocols and adjustments in amplification conditions, PCR-based studies of microbial community structure must be scrutinised carefully and are inherently compromised in their quantitative power (von Wintzingerode et al. 1997).

Interestingly, new advances in the screening of environmental libraries have brought the pre-PCR approach of microbial gene discovery back into vogue. Soil meta-genomes, cloned within either large- or small-insert libraries, can be directly screened for rRNA genes (Rondon et al. 2000; Venter

et al. 2004). It is now typically the goal in such studies to reveal the diversity of genes key to particular properties of interest (see Chap. 11), but such approaches can also provide PCR-independent surveys of rRNA gene diversity (Liles et al. 2003). Although these strategies may provide a community perspective free of PCR artifacts, they are still highly demanding on human and financial resources. As new screening strategies improve, this may become more feasible for standard analyses. Of course one must keep in mind that other biases such as preferential cloning (e.g. due to insert size) and biases in DNA isolation procedures (Frostegård et al. 1999; see Chap. 3) will still affect results. The latter is a general and far from trivial concern in soil community analysis, and there are numerous methods to liberate and isolate nucleic acids from soil samples (e.g. Griffiths et al. 2000; Hurt et al. 2001). Although manufactured kits have become the method of choice due to their convenience, it must be considered that most such kits have not yet been tested across a full range of organisms and conditions.

8.2.3 DNA Versus RNA Targets

The targeting of either RNA or DNA in microbial community analyses has important implications not only in the interpretation of results, but also in terms of methodological constraints. RNA is less stable than DNA, both in soil systems as well as during laboratory manipulation. In general, it is assumed that target DNA levels provide information concerning gene numbers in a sample, while RNA levels yield information relevant to gene activity. rRNA levels have been used as an indicator of general cell activity (Poulsen et al. 1993; Wagner et al. 1994), but the correlation between rRNA content and cell activity breaks down at low growth rates, which are typical of soil systems. Indeed, many soil-borne organisms have developed a strategy of maintaining high ribosome levels in times of dormancy so that they can become immediately active when environmental conditions dictate. Thus, although rRNA-based studies provide a different perspective than rDNA-based ones (Duineveld et al. 2001), one should not assume that one can extrapolate detected rRNA-based signals into cellular activity of the detected populations.

When comparing analyses of protein-encoding genes with those targeting the resulting mRNAs, it is generally accepted that transcript levels provide a decent approximation of the relative activity of the encoded function. Given this relationship and the rapid degradation of non-functional mRNA in the environment, analyses that target mRNA provide an opportunity to address the activity of genes, as opposed to simply their presence. Although mRNA extraction procedures can be tricky and are as yet not

validated with respect to efficiency, mRNA approaches are rapidly gaining in popularity (see Chap. 11). An even more direct functional assessment of microbial communities in soil might be feasible via protein-based approaches aimed at describing *in situ* protein diversity and function. Recent developments in environmental proteomics, an exiting and challenging field, are presented in Chaps. 4 and 5.

Non-active DNA, within dead or partially destroyed cells or free within the soil, can be stable for variable lengths of time. Although DNA stability in the environment is not known, it is highly influenced by the nature of the environmental matrix (see Chap. 7; Trevors 1996). Thus, it must be remembered that some of the recovered DNA could originate from organisms that do not have ecological significance in the sample at the time of sampling. On the other hand, one must also remember that RNA-based analyses using reverse transcription (RT)-PCR require an additional step in comparison to DNA-based PCR approaches, which may introduce additional biases.

8.2.4 Cloning Inventories Versus Community Profiling Methods

Two basic strategies have been applied, either separately or in parallel, to the analysis of complex mixtures of marker genes recovered from soil environments: the cloning approach and the community profiling approach. In general, cloning-based methods provide detailed information about the identity of numerous populations in a sample, but their demand on effort and resources generally limits their use to few samples. Thus, the cloning approach is particularly useful for obtaining sequence information concerning the populations in a sample of interest, which may be used for phylogenetic analyses and development of probes for specific community members. However, they are generally ill suited for comparison of larger numbers of samples, such as is necessary for the studies across environmental gradients. Profiling methods provide a more cursory inspection of the community, and allow simultaneous examination of multiple community members, which makes them well suited for the analysis of multiple environmental samples. The most commonly used community profiling methods include denaturing gradient gel electrophoresis [DGGE; note that for the purposes of this chapter we group other related gradient methods, TGGE (temperature) and TTGE (temporal temperature), under the single heading DGGE], terminal restriction fragment length polymorphism (T-RFLP) and single-strand conformational polymorphism (SSCP), but there are numerous other possibilities (see Johnsen et al. 2001; Stephen and Kowalchuk 2002). In all these methods, species-specific traits (typi-

cally sequence variation) are used to separate individual populations from complex mixtures of PCR products to provide a community fingerprint. These fingerprints can be compared across samples, and the development and application of numerical analysis methods has greatly increased the robustness of such comparisons (e.g. Fromin et al. 2002). In addition, tentative species assignments (or higher order taxon affiliations) can often be derived from detected populations by sequence analysis of excised bands or phylogenetic inference based on extant databases (for T-RFLP; see RDP-II hub at http://rdp.cme.msu.edu; Cole et al. 2003; Moyer et al. 1996). Ideally, one would use the sequence information derived from cloning and profiling surveys to return to the original samples to examine the size and physical location of each of all the microbial populations present in a sample, e.g. by fluorescent *in situ* hybridisation (FISH; Amann et al. 1995). Other quantitative methods to detect specific populations or groups *in situ* include quantitative PCR techniques or dot-blot hybridisation. However, it is clearly not feasible to follow such taxon-by-taxon approaches for the breadth of microbial diversity in soil, and these applications have necessarily been limited to specific populations of interest or broad microbial groups (Zarda et al. 1997).

Community profiling methods have come into fashion principally due to the fact that it has not been technically possible to process and sequence a sufficient number of clones to compare multiple samples via cloning-based approaches. This need for simplification may be partially overcome in the near future due to vastly expanding sequencing and computing capabilities and the development of microarray-based library screening methods (Valinsky et al. 2002a,b). Phylogenetic microarrays, so-called phylochips, may provide a tool to combine the highly detailed analysis of clone libraries with the high-throughput strategy of community profiling methods (see Chap. 9). Although several phylogenetic microarray platforms show great promise (e.g. Small et al. 2001; Loy et al. 2002), the technical challenges of such complex hybridisation experiments, the high costs still associated with microarray experiments, their limited quantitative capabilities and our incomplete knowledge of extant microbial diversity still inhibit their widespread use (Cook and Sayler 2003; Zhou 2003). To date, phylochip approaches work best when applied to habitats that have already been well characterised (i.e. the identity of most target populations is already known), and their general application at present can probably best be viewed as a high-end profiling method.

8.3 General Approaches for Microbial Community Description

8.3.1 The rRNA Approach

Ribosomal RNA genes, especially the small subunit (SSU) ribosomal RNA genes, have become the targets of choice for microbial ecologists involved in the *in situ* characterisation of soil-borne microbial communities. A number of properties of this marker have made it highly useful in such studies: (1) Its ubiquitous presence in all known organisms, and its lack of horizontal gene transfer between organisms (Sneath 1993); (2) conserved secondary structure, facilitating sequence alignment (Van de Peer et al. 1999); (3) presence of both highly conserved and variable regions, whose sequences are shared between broad and very narrow taxonomic groupings, respectively; (4) the high copy number of SSU rRNAs in the cell, facilitating detection; (5) appropriate size to contain large amounts of phylogenetic information; and (6) the exponentially expanding database of SSU rRNA gene sequences available for comparison (>60,000 sequences; http://rdp.cme.msu.edu/html; http://www.psb.ugent.be/rRNA/index.html).

One complicating factor of SSU rDNA as a target is the variable number of copies of rRNA operons per microbial cell. Although the growing database with respect to prokaryote rRNA gene copy number (http://rrndb.cme.msu.edu/rrndb/servlet/controller; Klappenbach et al. 2001) allows one to compensate partially for this factor, this can obscure the relationship between the number of recovered rDNA targets and the actual cell numbers in a sample. The conserved nature of the SSU gene and the conserved structure of its product RNA facilitate sequence alignments. However, the amount of discriminatory information for closely related species can be very limited, and rRNA sequence differences are not necessarily reflective of differences across other parts of the genome.

A number of primer systems have been developed for broad-level PCR-based community analysis approaches (Table 8.1). Several of these approaches have become quite standard, facilitating result comparisons across laboratories. Unfortunately, the majority of SSU rDNA sequences obtained to date have been recovered by PCR using supposedly 'universal' or 'domain-specific' primers that in reality do not possess the intended breadth of coverage (Watanabe et al. 2001b), thus biasing results accordingly. Such biases can be partially compensated by using multiple primer sets in a single study. It is assumed that different primers will not have the same biases, and corroborative data from multiple primer sets greatly increases the robustness of the analysis.

Table 8.1. Summary of commonly used PCR primer sets for domain level and fungal kingdom community analyses in soil

Primer pair	Target group	Position[a]	Comments	References
SSU rRNA-based primer pairs				
341f-GC (Muy-f)/518r (Muy-r)	Bacteria	341–535	First primers used in the application of PCR-DGGE to the study of microbial community structure. General bacterial primers. Produce short fragments.	Muyzer et al. 1993
341f-GC (Muy-f)/907r	Bacteria	341–924	Produces longer fragments than original primer set.	Muyzer et al. 1995
968f-GC/1401r	Bacteria	968–1401	General bacterial primers set (some groups are missed, i.e. Sphingomonas; Chang et al. 2000).	Nübel et al. 1996; Heuer et al. 1997
E8f/E1115r	Bacteria	8–1115	Used in cloning study of Octopus Springs to detect novel microbial lineages.	Reysenbach et al. 1994; Martinez-Murcia et al. 1995
E341f/E939r	Bacteria	341–939	Contain inosine residues to allow better coverage of bacterial domain. Primers described as bacteria-specific (Baker et al. 2003).	Watanabe et al. 2001a
E334f/E939r	Bacteria	334–939	Amplify same region as above.	Rudi et al. 1997
E9f/1512 r	Bacteria	9–1512	Primers described as bacteria-specific (Baker et al. 2003).	Hansen et al. 1998
63f-Cy5/783r	Bacteria	63–783	This primer combination reported to provide high number of OTUs in T-RFLP analyses of bacterial communities.	Marchesi et al. 1998; Sakai et al. 2004
RA46f/REA1100r followed by ARCH340f/ARCH519r	Archaea	46–534	Nested PCR for DGGE analysis.	Øvreås et al. 1997
7-forward/907-reverse	Archaea	7–907	Used for cloning and ARDRA analyses.	Giovannoni et al. 1988; Jurgens et al. 1997

Table 8.1. (continued)

Primer pair	Target group	Position[a]	Comments	References
UA571F/ UA1204R	Archaea	571–1204	16S rDNA libraries from thermophilic communities.	Baker and Cowan 2004
UA751F/ UA1406R	Archaea	751–1406	16S rDNA libraries from thermophilic communities.	Baker and Cowan 2004
NS1f/ITS4r	Fungi	5′-end of 18S to 5′-end of 28S rRNA genes	General fungal primers set to amplify large fragment containing full 18S rRNA gene, ITSI & II and 5.8S gene; employed in T-RFLP analysis	White et al. 1990; Dickie et al. 2002
NS1f-GC/ NS2r+10	Fungi[b]	17–583	NS2+10r overlaps the site of NS2r (White et al. 1990). Primer set used for DGGE.	Kowalchuk et al. 1997a
EF4f/EF3r	Fungi	195–573	EF4f-EF3r to amplify mainly Basidiomycota and Zygomycota. EF4f combined with Fung5r amplify mainly Ascomycota. EF3r points in the reverse direction, near the 3′-end of the 18S rRna gene.	Smit et al. 1999
EF4f/518r-GC	Fungi	195–301	Recovers short fragment from most fungi.	Schabereiter-Gurtner et al. 2001
NS26f/518r-GC	Fungi	305–581	Region contains limited amount of phylogenetic information.	Schabereiter-Gurtner et al. 2001
NS2f/Fung5r-GC	Fungi	337–747	Generally used to produce amplicons for separation via DGGE.	Van Elsas et al. 2000
NS1f/FR1r-GC	Fungi	17–1664	General fungal primer set produces 1650 bp rDNA fragments. Used for DGGE analysis despite large size.	Vainio and Hantula 2000
FF390f/FR1r-GC	Fungi	1317–1664	General fungal primer set, produces 390 bp rDNA fragments.	Vainio and Hantula 2000
NS1f/GC-fungr	Fungi	20–368	Used to examine fungal communities in silage.	May et al. 2001
Other targets				
LSU rRNA (U1r/U2-GCf)	Fungi	403–662	Primer set amplifies 260 bp fragments from a variable region of fungal 28S rRNA gene.	Möhlenhoff et al. 2001

Table 8.1. (continued)

Primer pair	Target group	Position[a]	Comments	References
Internal transcribed spacer (ITS1f/ ITS4-GCr)	Fungi	Fungal internal transcribed spacer (ITS) II region	ITS4 points into ITS II region at the 5′-end of 28S gene. ITS4-GCr can also be combined with ITS1f-XbaI (ITS1 + Xba I site).	White et al. 1990; Klamer et al. 2002
rpoB (*rpoB*1698f/ *rpoB*2041r)	Bacteria	–	The RNA polymerase β-subunit (rpoB) offers an alternative to the 16S rRNA gene for community analyses based on DGGE.	Dahllöf et al. 2000; Peixoto et al. 2002
gyrB (UP-1/UP-2r)	Bacteria	–	Universal primers based on conserved regions of the subunit B protein of DNA gyrases.	Yamamoto and Harayama 1995
*rec*A (not named)	Bacteria	–	Amplify the internal region of the *atp*A, *car*A and *rec*A genes. Primer set originally applied for the detection and taxonomic analysis of the bacterial genus *Pseudomonas*.	Hilario et al. 2004

[a] According to *E. coli* for prokaryotic targets, with respect to *Saccharomyces cerevisiae* 18S rDNA for eukaryotic targets.
[b] Not specific only to fungi, also target other eukaryotes.

8.3.2 Other General Markers for In Situ Determinations of Microbial Community Structure

Despite the attractiveness and usefulness of the SSU gene as target, sole reliance on this marker clearly can give an incomplete, limited and/or biased view of microbial community structure. The use of other suitable broad-level phylogenetic markers can provide a more balanced community assessment. To be reliable, alternative phylogenetic markers for microbial community description should fulfill the same prerequisites that make SSU rRNA gene sequences so useful, i.e. wide distribution, genetic stability, phylogenetic power and a comparative database. Genome comparisons reveal that the vast majority of genes are not ubiquitously present across all organisms, so that the number of potential broad-purpose markers is

rather limited. No marker has a database approaching the one that is now available for SSU rRNA genes, but several good candidates that have received attention include the genes encoding the large subunit (LSU) rRNA, components of RNA polymerase (e.g. *rpoB*), topoisomerases (e.g. gyrB; Watanabe et al. 2001b), elongation factors G (EF-G) and Tu (EF-Tu), F_1F_0 ATP synthase β-subunit (*atpD*), the RecA protein (*recA*), and the HSP60 heat shock protein (Sigler et al. 1998), as well as the internal transcribed spacer (ITS) regions of the rRNA operon. In general, the phylogenies predicted by these markers match well with those produced using SSU data, thus corroborating the validity of most SSU-based analyses (Ludwig et al. 1998). However, SSU sequences cannot resolve all phylogenetic issues, nor can they identify all microbial species unambiguously. Given these limitations, and the inherent danger in relying on a single molecular marker, further development of additional complementary broad-purpose markers should be a research priority. Modern high-throughput sequencing capabilities and future genome comparisons should help to bolster databases of alternative markers and identify those that are best suited for analysing complex microbial communities, such as those inhabiting soils.

Other rRNA-Related Community Analysis Markers

The LSU rRNA molecule offers many of the same advantages as listed above for SSU rRNA genes, and actually contains more phylogenetic information, but has been used less frequently in community analysis studies, partly due to its larger size. Consequently, a less extensive database for LSU rRNA gene sequences is currently available. The other product encoded within the rRNA operon, the 5S rRNA (or 5.8S in eukaryotes), has also been used to describe microbial communities, but its small size limits its use as a phylogenetic marker. In addition to RNA-encoding genes, rRNA operons also typically contain so-called ITS regions. Because these regions do not encode structural products, they accumulate mutations rapidly, making them ideal for the comparison of closely related species or even populations within a species (Garcia-Martinez et al. 1999). However, the lack of structural homology across taxa makes broad comparison of sequence information based upon this marker difficult or impossible.

General Non-rRNA Markers Used for Microbial Community Analysis

Protein-coding genes can share some or all of the advantageous characteristics of rRNA gene-based targets and can also have additional benefits. Since most amino acid sequences of proteins are derived from the nucleotide sequences of their structural genes, two levels of information are available for subsequent analyses, i.e. nucleotide or amino acid sequence. Also, the degree of resolution and phylogenetic information can greatly surpass that

of SSU rRNA sequences, due to accumulation of neutral nucleotide substitutions at protein-coding positions that do not alter the subsequent amino acid sequence. The high substitution rates at such wobble positions facilitate the comparison of closely related species. In many eukaryotic genes, intron regions, which are highly susceptible to mutation, can also act as highly variable markers. Conversely, substitution saturation problems (i.e. when substitution frequencies per site are too high to be informative) in distantly related strains can often be eliminated by translation to amino acid sequences. Furthermore, basic structural integrity of protein motifs facilitates sequence alignment. An additional advantage of most protein-encoding genes is that they exist as single copy genes in microbial genomes, simplifying the extrapolation of recovered sequence information to population sizes in the original sample.

Similar to rRNA genes, most protein-encoding genes, and their resulting proteins, contain domains that vary in their level of conservation across taxa. Amino acids involved in active site formation and other essential protein motifs are highly conserved, whereas other portions of the genes are under far less stringent selection. Thus, (degenerate) primers targeting conserved regions can often be designed to amplify the marker gene from a broad range of organisms, yielding products that contain a large degree of variation in the intervening sequence. The *rpoB* gene has recently been suggested as a suitable alternative to SSU rDNA-based analyses of microbial communities (Dahllöf et al. 2000). To date, the majority of studies using alternative broad-range microbial markers have focused on the phylogenetic analysis of genes recovered from isolated organisms. However, given the capacity to engineer conserved primers for environmental study (e.g. Yamamoto and Harayama 1995), there is no reason why similar strategies should not be devised and implemented for other markers. Also, the utility of such analyses will increase significantly as databases for alternative community analysis markers continue to grow (e.g. Watanabe et al. 2001b). It should be noted that molecular phylogeny deduced from a single locus may be unreliable due to the stochastic nature of base substitutions or rare horizontal gene transfer events. Thus, multiple-marker analyses always provide more robust information than those relying on a single gene target.

8.4 Group-Specific Microbial Community Analyses

As stated earlier, tackling the breadth of microbial diversity in soil is a complicated and daunting task. To reduce the problem to manageable levels, many researchers have chosen to limit their analyses to specific subsets of the entire community, because these subsets play a crucial role in the func-

tioning of that community. These studies either combine several group-specific approaches to obtain a piecemeal picture of the community as a whole (e.g. Boon et al. 2002), or they limit their focus to a particular group, or groups, of interest.

In principle, group-specific primer sets are designed such that all members of the target group are amplified by the combined specificity of the two primers, or by a nested PCR primer approach. However, the specificity of such approaches is a function of the matching of primers with specific target sequences, which invariably cannot be perfectly designed based on available sequence information. Thus, one must keep in mind that group-specific approaches are only as robust as the primer sets used and confirmation that detected populations are actually from the desired target group (i.e. by sequence or probe analysis) greatly increases the robustness of the data.

8.4.1 rRNA Approaches Focused on Specific Phylogenetic Groups

The presence of SSU rDNA domains with varying levels of conservation across taxa facilitates the design of primers and probes that target specific microbial groups at various taxonomic levels. Thus, conserved regions may be used for the design of high level taxon primers (see above; Table 8.1), intermediately variable regions may be applied to analyses at the order through class level, and highly variable regions are suited for detection of specific genera and species (e.g. Heuer et al. 1999). Numerous primers designed to target intermediate level phylogenetic groupings have been utilised for the development of group-specific community analyses (Table 8.2). Such group-specific approaches fall into two categories: (1) those targeting phylogenetic groups comprised of numerous functional groups and (2) those targeting phylogenetic groups that also correspond to functional groups. In many cases, rather closely related microorganisms can belong to very different functional groups. Thus, the majority of analyses that target intermediately broad microbial groups will detect a range of functionally distinct populations. One can say little about an organism's function based on its phylogenetic classification as a member of, for instance, the α-subclass of the Proteobacteria or the Actinobacteria. Thus, while such group-specific approaches provide highly valuable tools to delve into manageable portions of soil microbial diversity, they do not necessarily bring us much closer to describing the function of the microbial populations detected. In some cases, however, specific functions are limited to a narrow defined range of microbial taxa. Examples include ammonia oxidation, methane oxidation, sulfate reduction and arbuscular mycorrhiza

Table 8.2. Selection of group-specific microbial community analysis approaches based on rRNA gene markers

Target group	Primer names	Types of analyses	Comments	References
Phylogenetic groups				
Actinobacteria	F243-R1378	Nested PCR, (T)DGGE	Specificity comes from forward primer, which is based on a probe that has been used to detect this group.	Heuer et al. 1997
α-subclass Proteobacteria	F203a-R1378	Nested PCR, (T)DGGE	Specificity comes from forward primer, which is based on a probe that has been used to detect this group.	Heuer et al. 1997; Gomes et al. 2001
β-subclass Proteobacteria	F948β-R1378	TGGE	Specificity comes from forward primer, which is based on a probe that has been used to detect this group.	Heuer et al. 1997; Gomes et al. 2001
Verrucomicrobia group	I-341fGCf-I533r	DGGE	Inosine residues included to gain greater coverage of all bacterial groups and reduce amplification biases caused by mismatches in 16S rDNA.	Watanabe et al. 2001a
Acidobacterium group	31f -R1378	Nested PCR, DGGE	Allowed identification of a novel lineage within the kingdom in a hot spring habitat; it can also be applied to soil systems.	Barns et al. 1999
Pseudomonads	Ps-for (S-G-Psmn-0289-a-A-18)/Ps-rev (S-G-Psmn-1258-a-A-18)	Cloning, MERFLP	Specific for *Pseudomonas* (sensu stricto).	Widmer et al. 1998; Porteous et al. 2002; Garbeva et al. 2004
Burkholderia	Burk3f-BurkR-GC	DGGE	8% of the *Burkholderia* sequences in database did not match the forward primer sequence perfectly.	Salles et al. 2002

Table 8.2. (continued)

Target group	Primer names	Types of analyses	Comments	References
Lactic acid bacteria	S-G-Lab-0677-a-A-17(Lab-0677r)/S-G-Lab-0159-a-S-20 (Lab-0159f)	DGGE	The primer set selectively amplifies 16S rDNA from lactobacilli and related acid bacteria, including members of the genera *Leuconostoc*, *Pediococcus* and *Weissella.*	Heilig et al. 2002
Bacillus	BacF- R1378	DGGE	Both specific forward primers can be coupled with universal reverse primer.	Garbeva et al. 2003
Paenibacillus	PAEN515f/ R1378	Cloning, nested PCR, DGGE	Targets several species within the genus *Paenibacillus*	da Silva et al. 2003
Basidiomycetes	ITS1f – ITS4B	Cloning	Fungal-specific forward primer with Basidomycete-specific reverse primer.	White et al. 1990; Gardes and Bruns 1993; Rosling et al. 2003
Phylogenetic "functional" groups				
Ammonia-oxidising β-subclass Proteobacteria	CTO189f-CTO654r	DGGE	Seven distinct sequence clusters within the genera *Nitrosospira* and *Nitrosomonas* were detected.	Kowalchuk et al. 1997b
Type I and II methanotrophs	MB10γ-533r and MB9α-533r	DGGE, cloning	The 16S rRNA analysis was coupled with functional targets (see Table 8.3).	Henckel et al. 1999
Methanogenic Archaea	0357F-0691R	DGGE	Primers amplified non-methanogenic archaea in pure culture but they were not detected in DGGE.	Watanabe et al. 2004
Nematodes	NEMF1-NEM896r	DGGE, cloning	It was applied successfully to the identification of nematodes in a range of European grasslands.	Waite et al. 2003

Table 8.2. (continued)

Target group	Primer names	Types of analyses	Comments	References
Kinetoplastida (Protozoa)	Kin24SF-Kin24SR	DGGE, cloning	Target the α-subunit of the 24S rRNA.	Rasmussen et al. 2001
Cyanobacteria	CYA359F-CYA781R	DGGE	It was also tested successfully on symbiotic cyanobacteria in lichens.	Nübel et al. 1997
Arbuscular mycorrhizal fungi	FLR3-FLR4	Cloning	Targets LSU rDNA.	Gollotte et al. 2004
Arbuscular mycorrhizal fungi	NS31-AM1	DGGE, cloning	Discrimination at the genus to species level; uncertainty about the amplification of all Glomeromycota.	Helgason et al. 1998; Kowalchuk et al. 2002
Gigasporaceae	FM6-GIGA5.8R	DGGE	Results showed a high intraspecific heterogeneity.	de Souza et al. 2004

(see Table 8.2). In analyses targeting such phylogenetic/functional groups, it is assumed that all detected populations that fall within the phylogenetic group of study share the physiological feature of that functional group. The discovery of organisms within these clades that do not possess the function in question serves to undermine this assumption, and it cannot be ruled out that other yet-to-be-discovered organisms outside the phylogenetic group in question share the same functional trait, making the analysis incomplete.

8.4.2 Specific Approaches Based on Specialised Functional Genes

The majority of microbial functional groups are polyphyletic, limiting the applicability of rRNA-based approaches. Thus, while phylogenetic marker approaches to microbial community analysis provide tools to determine which organisms are present or generally active in a community, they provide little insight into the functions carried out by the detected organisms, except in the few cases where function can be inferred from phylogeny. A more function-oriented approach is to target specific genes that are distinctive of a particular microbial functional group, i.e. genes responsible for the defining phenotype of the functional group. Suitable functional gene targets are homologous across the breadth of the functional group and absent in all organisms outside of the functional group. Some compromises are often necessary due to common ancestry of genes that encode different protein functions, such as the overlapping ancestry of genes encoding methane and ammonia oxidation (Nold et al. 2000). Still, a number of such approaches have been recently developed to describe the genes and organisms responsible for key soil functions, such as nitrogen, carbon, and sulfur transformations, degradation of specific compounds and antibiotic producers (Table 8.3).

It is important to note that even such specific functional gene approaches still use these genes simply as markers to detect different genotypes, as is the case in general marker approaches. Such analyses are designed to detect genotypic variation between target organisms, but this variation cannot be translated into population differences at the phenotypic level. In the vast majority of cases, the differences that convey phenotypic variation between functional group members lie outside the genes targeted for their detection. Thus, similar to other community analysis approaches, 'functional gene markers' only provide information concerning the distribution (DNA-based approaches) or transcript levels (mRNA-based approaches) of specific gene populations across environmental gradients. Furthermore, it is not always possible to infer the phylogeny of organisms based on the

Table 8.3. Selection of microbial community analysis approaches targeting group-specific structural/functional genes

Function targeted	Gene targeted	Target details	Types of analyses	References
Nitrogen cycling				
Ammonia oxidation	amoA	β-subclass proteobacterial ammonia monooxygenase gene	Cloning, T-RFLP, DGGE	Rotthauwe et al. 1997; Stephen et al. 1999; Horz et al. 2000; Avrahami et al. 2002; Nicolaisen and Ramsing 2002
Nitrogen fixation	nifH	Bacterial dinitrogenous reductase gene	DGGE, cloning	Diallo et al. 2004; Rösch et al. 2002
Nitrous oxide reduction	nosZ	Bacterial nitrous oxide reductase gene	Cloning	Rösch et al. 2002
Denitrification	nirK and nirS	Bacterial nitrite reductase gene	Cloning	Braker et al. 1998; Rösch et al. 2002
Carbon cycling				
Methane oxidation	pmoA and mxaF	Bacterial methane monooxygenase and methanol dehydrogenase genes	Cloning, DGGE	McDonald and Murrell 1997a,b; Henckel et al. 1999
Lignin-related aromatic compounds degradation	pcaH	Bacterial ring-cleaving oxygenase gene	Cloning	Buchan et al. 2001
Phenol degradation	LmPH	Bacterial phenol hydroxylase gene	TGGE	Watanabe et al. 1998
Microbial interactions/plant pathology				
Lignin degradation	Laccase	Ascomyceteous laccase gene	Cloning	Lyons et al. 2003
2,4-Diacetylphloro-glucinol production	phlD	Bacterial polyketide synthase of the DAPG operon	PCR detection, RFLP of mixed samples	Raaijmakers et al. 1997; Gardener et al. 2001
Chitin degradation	chiA	Bacterial chitinase gene	DGGE, cloning	Williamson et al. 2000; Metcalfe et al. 2002
Others				
Sulphate reduction	[NiFe] hydrogenase	Two targets are described: *Desulfovibrio* spp. and Bacteria	DGGE	Wawer and Muyzer 1995
Sulphate reduction	DSR	Bacterial sulfite reductase gene	Cloning	Castro et al. 2002

phylogenetic analysis of specific functional markers, especially those that are prone to horizontal gene transfer, such as those encoding peripheral catabolic pathways and antibiotic resistance (Thomas 2000). One is typically not able to assign an organism to the genes recovered, unless the functional gene and a phylogenetic marker can be recovered on a contiguous piece of DNA (Beja et al. 2000).

Important developments in this area are the design of new community profiling methods for an increasing suite of specific functional genes (see Table 8.3), the implementation of more quantitative PCR and RT-PCR approaches, and the multiplex analysis of large numbers of genes via functional gene microarrays (see Chap. 9; Wu et al. 2001; Rhee et al. 2004).

8.5 Concluding Remarks

This chapter has attempted to outline the decisions that need to be made when choosing and applying methods to analyse the structure of microbial communities in soil. With the explosion of molecular studies of soil-borne microbial communities in the past decade, it would have been impossible to review all of the advances in this field here. Instead, our goal was to describe the critical trends, possibilities and limitations of various approaches so as to illustrate how the choice of technique is dictated by the question being asked, the type of information required and the resources available. Before applying these evolving molecular tools, one must first realise the difference between analyses of community structure versus actual microbial diversity. Also, the pro's and con's of using PCR as a basis of target amplification need to be weighed seriously. In total, it is clear that there is no single 'holy grail' when it comes to microbial community analysis approaches, and the application of multiple approaches will invariably lead to more robust results than any single approach.

Molecular microbial community analyses have become commonplace in today's modern microbial ecology laboratory. However, these methods are in a continuous state of improvement, adaptation and expansion. Trends for today and tomorrow include:

- Further development and validation of primer- and probe-based analyses.
- Expansion of databases for alternative microbial markers.
- Further advancement in analytical tools to assess structure and significant trends in molecular inventory and community profile data.
- New high-throughput sequencing possibilities and microarray approaches (see Chap. 9) which hold the possibility to bridge the gap between highly detailed studies and analysis of multiple samples.

- Application and further development of methods that link community structure to microbial functions in soil (Gray and Head 2001; Nannipieri et al. 2003). Such methods include stable isotope probing approaches (see Chap. 10) and methods that couple microbial identification with substrate uptake (e.g. Micro-FISH/STAR-FISH and array probing).

Combining our molecular arsenal that has developed over the last two decades with insightful experiments and environmental analyses should help us to unravel the structure and function of the soil-borne microbial communities that are so critical to the functioning of terrestrial ecosystems.

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9 Advances in Microarray-Based Technologies for Soil Microbial Community Analyses

Christopher W. Schadt, Jizhong Zhou

9.1 Introduction

Studies on the diversity of microbial life within soils indicate that there are several thousand distinct microbial genome types present per gram of soil (Torsvik et al. 1990, 1996), and efforts to further characterise this diversity have revealed significant numbers of previously unknown organisms within the bacterial, archaeal and eukaryal domains of life (Bintrim et al. 1997; Hugenholtz et al. 1998; Dawson and Pace 2002; Schadt et al. 2003; Zhou et al. 2003). Microbial communities in soils also often exhibit extreme spatial and temporal variability; however, the factors that underlie and influence this variation remain largely unknown (Zhou et al. 2003). Perhaps one reason that this goal has remained elusive is a lack of common methodologies that allow for rapid, inexpensive and comprehensive characterisation of these extremely high levels of diversity.

The application of microarrays to the study of gene expression in microbial genomics now regularly allows for comprehensive, high-throughput studies of organisms under a variety of physiological conditions (e.g. Lockhart et al. 1996; Schena et al. 1996; DeRisi et al. 1997; Ye et al. 2000; Thompson et al. 2002; Liu et al. 2003) and this technology is becoming cheaper and easier to implement. Currently, the adaptation of such techniques to problems of microbial ecology in soils and other environments is a major focus effort that is taking place in many research groups. However, the adaptation of these technologies for the detection of microbial genes in complex environmental samples presents many unique challenges (Zhou and Thompson 2002; Zhou 2003; Schadt et al. 2005). This chapter reviews the progress of those efforts to date with an emphasis on the potential application of this technology to questions in soil ecology.

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9.2 Types of Environmental Microarrays

Based on the type of probes arrayed and their potential applications, Zhou and Thompson (2002) classified microarrays of potential use in environmental studies into three different basic groups: (i) *Phylogenetic oligonucleotide arrays* (POAs) primarily contain sequence probes derived from rRNA genes. These types of arrays might be used for the analysis of microbial community composition and structure under a phylogenetic framework (e.g. Loy et al. 2002; Valinsky et al. 2002a,b; El Fantroussi et al. 2003). (ii) *Functional gene arrays* (FGAs) contain probe sequences for genes encoding key proteins involved in various environmental processes such as steps in denitrification or the degradation of naphthalene. These arrays are useful for monitoring the physiological status and functional activities of microbial communities in natural environments (e.g. Wu et al. 2001; Taroncher-Oldenburg et al. 2003; Rhee et al. 2004). (iii) *Community genome arrays* (CGAs) are constructed using whole genomic or large fragment DNA isolated from pure culture microorganisms or even large fragments of genomic DNA originating from fosmid or BAC libraries. These arrays can be useful to describe a microbial community in terms of its cultivable component or screen isolates and metagenomic libraries for discovery of target genes and organisms (e.g. Cho and Tiedje 2001; Wu et al. 2004).

9.3 Important Issues in Microarray Analysis

9.3.1 Specificity

Although gene-based oligonucleotide microarrays have been used successfully for monitoring genome-wide gene expression, the use of POAs and FGAs with complex environmental samples presents unique technical challenges in terms of specificity. The highly conserved nature of the rRNA, and the often high degree of similarity among homologous and orthologous functional genes, makes specific probe design much more difficult to achieve. Additionally, especially in the case of rRNA, the hybridisation of oligonucleotide probes to target nucleic acids possessing stable secondary structure can be particularly challenging. Stable secondary structure of target DNA or RNA must be overcome in order to make complementary sequence regions available for duplex formation with probes and, for this reason, these regions should be avoided. Thus the stable secondary struc-

ture of rRNAs can have serious effects on hybridisation specificity and detection sensitivity.

The potential advantage of oligonucleotide probes is that target sequences containing single base mismatches can be differentiated by microarray hybridisation. However, in reality, with the high number of possible probe and target combinations, this has been difficult to systematically demonstrate. To systematically determine whether single mismatch discrimination can be achieved for 16S rRNA genes using microarray hybridisation, a model oligonucleotide microarray consisting of probes derived from three different regions of the 16S rRNA molecule corresponding to different bacterial taxa was generated in our laboratory (X. Zhou and J. Zhou, unpubl. data). The probes had 1–5 mismatches in different combinations along the length of the oligonucleotide probe with at least one mismatch at the central position. Hybridisation signal intensity with a single-base mismatch was decreased by 10–30% depending on the type and position of the mismatched nucleotide base. The signal intensity of probes with two base mismatches was 5–25% of that of the perfect match probes. Probes with three or four base-pair mismatches yielded signal intensities that were 5% of that of the perfect match probes. Maximum discrimination and signal intensity was achieved with 19-base probes. These results indicated that single base discrimination for small subunit (SSU) rRNA genes can be achieved with glass slide-based array hybridisation, but complete discrimination appears to be problematic with SSU rRNA genes and most likely not possible using only single mismatches. Letowski et al. (2004) recently published similar studies showing optimal discrimination was achieved with mismatches distributed nearly evenly across the oligos. Many others have reported similar findings (Liu et al. 2001; Loy et al. 2002). This incomplete discrimination presents great difficulties when DNA derived from diverse, uncharacterised environmental samples is hybridised against POAs and FGAs. Interpretation of signals will obviously be confounded by the fact that, in environmental samples, lower than expected signals could be due either to low abundance of the target or imperfect matches between probe and target.

Compared to the short oligonucleotides commonly used for rDNA in POAs, the protein-coding genes used in FGAs are often designed using longer probes (40–70mer). While these probes may provide less hybridisation specificity, they provide better sensitivity levels. Additionally, the higher nucleotide variability of such genes often allows for species-specific design even at such probe lengths. Our recent studies indicated that genes having less than 88% nucleotide sequence identity could most often be discriminated with FGAs under hybridisation conditions of high stringency (50% formamide and 50 °C; Rhee et al. 2004). Additionally, a further characterisation of probe specificity in our laboratory has shown that by

limiting non-target hybridisation free energy to > −35 kcal/mol and limiting long stretches of continuously matched non-target bases to less than 20 bp can provide for further predictability in specificity, when considered simultaneously with percent similarity. Taroncher-Oldenburg et al. (2003) have reported similar results for 70mer oligonucleotide FGAs designed for nitrogen cycle genes.

Our past results have also shown that DNA/DNA hybridisation on CGAs in the presence of 50% (v/v) formamide at 55 °C could discriminate between microbial genomes of different species within a genus, whereas genomes, in many cases, could not be clearly distinguished at the subspecies level (Wu et al. 2004). By raising the hybridisation temperature to 65–75 °C, CGA-based discrimination between closely related bacterial strains could be achieved. Although some genes within bacterial genomes, such as rRNA genes, are highly similar even among different strains or species, the presence of such highly conserved sequences does not appear to affect the overall hybridisation specificity in CGAs.

9.3.2 Sensitivity

Since very small hybridisation volumes are used in microarray experiments, it is generally thought that the sensitivity of microarray hybridisation is higher than that of conventional membrane-based hybridisation. However, the sensitivity of hybridisation with glass-based microarrays may still be on the order of 100- to 10,000-fold less than with polymerase chain reaction (PCR) amplification and 100-fold less than with membrane-based hybridisation (Taniguchi et al. 2001). One of the main reasons that the sensitivity of glass-based microarray hybridisation is lower than membrane-based hybridisation could be because the probe-binding capacity on glass surfaces is much lower than that on porous membranes (Cho and Tiedje 2001). Increasing the DNA-binding capacity of glass slide microarrays could be one way to enhance microarray hybridisation sensitivity, and efforts to accomplish this using new types of chemical compounds for coating glass slides are ongoing.

Often detection limits are assessed using a known amount of target DNA for a given probe and progressively reducing target DNA concentration until the signal cannot be distinguished reliably from background fluorescence [e.g. signal-to-noise ratio (SNR) <2]. Using a 50mer biodegradation FGA and temperature of 50 °C in the presence of 50% formamide, Rhee et al. (2004) showed gene-dependent detection sensitivities to be between 5–10 ng in the absence of other DNA. However, DNA targets of environmental samples are present in a complex background of non-target DNA.

When assessed in a background of 2 μg of non-target *Shewanella* DNA, it was found that the detection limit for the same genes was about 10 times lower (~50–100 ng for a range of gene/probe targets). Assuming 4 Mbp genome size, this corresponds to a detection limit of $\sim 10^7$ cells being required for a gene to be detected in an environmental sample. These results correspond well with other published studies of detection limits. Using *nir*S gene PCR products as probes about 1 ng of labeled pure genomic DNA in the absence of background DNA could be detected (Wu et al. 2001). With PCR product targets of 500–900 bp, Cho and Tiedje (2002) showed detection limits to be ~10 pg in the presence of background DNA. The detection limit of CGAs is much lower and was estimated to be ~0.2 ng with pure labeled genomic DNA in the absence of background DNA, and about 5 ng of genomic DNA or 2.5×10^5 cells in the presence of background DNA (Wu et al. 2004). While such limits should be sufficient for detection of at least the more dominant members of a microbial community, this is still not sensitive enough to detect less abundant microbial populations in complex communities. To detect rare populations in natural environments, other approaches for increasing hybridisation sensitivity are needed. Our and other groups are currently exploring ways to enhance the levels of detection sensitivity.

9.3.3 Quantitation

We have evaluated the quantitative potential of microarray hybridisation for both FGA (Wu et al. 2001; Rhee et al. 2004; Tiquia et al. 2004) and CGA formats (Wu et al. 2004). Linear quantitative relationships are most often observed between signal intensity and target DNA concentration over concentration ranges from 1–1,000 ng for both pure cultures and mixed target DNA populations. These results suggest that DNA microarrays may potentially be used for quantitative analysis of environmental samples. However, the difficult challenge in quantifying microbial populations in natural environments is that the community composition of the environmental samples is largely unknown. It is assumed that hybridisation signal intensity is directly related to population abundance of the target organism. However, non-specific hybridisation due to unknown diverse members in a sample may contribute to signals.

One way to assess quantitative accuracy of microarray hybridisations is the use of real-time PCR with sequence-specific primers. While it is not possible to verify an entire large microarray data set because these techniques are labour and time intensive, verification of microarray findings for several key genes is achievable and is common practice in microbial

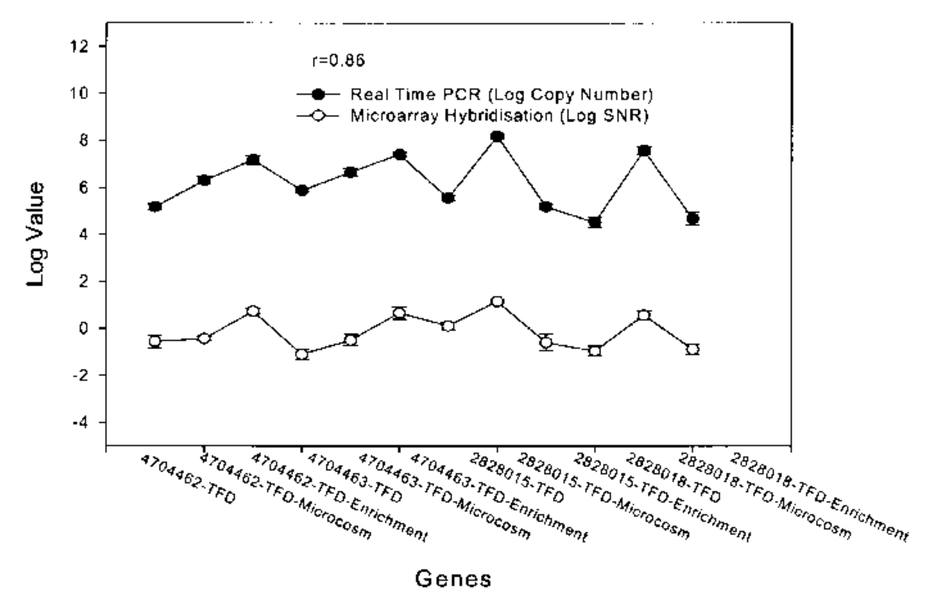


Fig. 9.1. Correspondence of microarray-based hybridisation signals with quantitative PCR for genes associated with naphthalene degradation (Rhee et al. 2004). Quantitative PCR may be used for independent verification of key results associated with microarray studies

gene expression studies. Rhee et al. (2004) used such an approach to verify microarray hybridisation results associated with naphthalene degradation (Fig. 9.1). Overall, significant correlations between the microarray hybridisation signals and the gene copy number determined by real-time PCR were obtained with $r^2 = 0.74$ for all genes tested, and $r^2 = 0.96$ for the genes with SNR > 3. Such results showing consistency between microarray hybridisation data and real-time PCR data are encouraging for the use of microarray data in quantitative interpretations.

9.4 Applications of Different Formats of Microarrays

9.4.1 Phylogenetic Oligonucleotide Arrays (POAs)

POAs primarily contain probes/sequences derived from rRNA genes; however, efforts have been made to incorporate other markers such as gyrB (Fukushima et al. 2003; Kakinuma et al. 2003) or rpoB (Dahllöf et al. 2000). The probes incorporated into POAs may be designed on a species-specific basis, a hierarchical manner, or some combination thereof, such

that several different taxonomic or phylogenetic levels of information are targeted. While species-specific probes can be easily generated on a one-by-one basis using relatively computationally simple algorithms or searches such as those available through the ribosomal database project (RDP; Cole et al. 2003), ARB (Ludwig et al. 2004) and ProbeBase (Loy et al. 2003), comprehensive and effective hierarchical probe design remains more difficult. These efforts require full and accurate alignments as well as robust phylogenetic information in order to accurately target specific groups of organisms and predict hierarchical probe behaviour. However, recently, significant progress has been made towards these fundamental issues. Using new search methods and quality score calculations, Zhang et al. (2002) were able to identify 10,487 perfect-match 15–25mers that together covered more than 86% of the phylogenetic nodes in a representative bacterial tree that included 929 operational taxonomic units (OTUs). This represented a core set from the RDP of highly reliable and complete sequences. Additionally, a new potential resource for comprehensive probe design called CASCADE-P is also under development (http://greengenes.llnl.gov/16S/). Through the incorporation of new global alignment algorithms (DeSantis et al. 2003), this group hopes to offer comprehensive data mining and probe design tools to the scientific community in the near future. While still under evaluation, these tools have already been used to build a 500,000 feature Affymetrix microarray containing sets of hierarchical probes (up to 25) for each OTU in the RDP database. Such phylogenetic arrays, once fully developed and evaluated, will be extremely useful for the analysis of microbial community composition and structure under a phylogenetic framework. Additionally, these methods offer much more quantitative potential than PCR-based methods. Unfortunately, because of low sensitivity levels, at present most studies still rely on a pre-hybridisation PCR amplification. This is feasible with POAs because of the highly conserved nature of the targeted 16S rDNA, but greatly complicates the quantitative interpretation of results due to the potential for biases in PCR amplifications. Additionally, as expression of rRNA has been shown to be highly correlated with growth rate and activity level (Asai et al. 1999a,b), rRNA targets could potentially also provide information on relative activity levels of the different target organisms on POAs if the sensitivity of hybridisations could be improved.

The actual application of POAs to the study of microbial ecology has come thus far from only a few example studies. While no comprehensive studies have yet been published from any environment, several groups have made great progress in targeting specific groups of organisms. For example, Loy et al. (2002) have developed a POA that includes probes claimed to distinguish all known sulfate-reducing lineages based on 16S rDNA sequences. In evaluative tests, this array of 132 probes was able to distinguish at least 41 species of sulfate-reducing taxa. In some cases these probes were

specific at even the strain and isolate level. Additionally, in application to mixed environmental samples from periodontal and hypersaline mat communities, this array returned results suggesting the presence of dominant taxa that were consistent with results from PCR-based studies targeting 16S rDNA and dissimilatory sulfite reductase (dsrAB) genes. Another recent study used a promising new combination of techniques to track active ammonium-oxidising bacteria in activated sludge samples (Adamczyk et al. 2003). The authors combined isotope labeling to track ammonia-oxidising bacteria incorporating ^{14}C-labeled bicarbonate, with a POA for the detection of specific rRNA associated with these organisms. The total rRNA in the sample was then labeled with Cy3 dyes, and, by using sequential fluorescent and autoradiographic scans of the resulting hybridizations, the authors could estimate the proportion of the population that was actively incorporating the labeled bicarbonate. Both of these studies demonstrate the promise of array technologies. However, both are extremely limited in depth of application and do not fully realise the potential of microarrays for comprehensive, high-throughput analyses.

9.4.2 Functional Gene Arrays (FGAs)

FGAs contain genes encoding key proteins involved in various environmental processes. Microbial involvement in processes important to ecosystem biogeochemistry such as nitrification, denitrification and sulfate reduction in ocean sediments (Wu et al. 2001; Tiquia et al. 2004), nitrogen fixation in picoplankton communities (Jenkins et al. 2004; Steward et al. 2004), and naphthalene degradation in soils (Rhee et al. 2004) have recently been targets of studies using FGAs. Our laboratory has been actively developing FGAs designed to be comprehensive for the known diversity of microbial functional genes involved in N, C (including methane) and S cycling, as well as genes involved in the biodegradation of organic contaminants and in the resistance to metal toxicity (Wu et al. 2001; Zhou 2003; Rhee et al. 2004; Schadt et al. 2005; Tiquia et al. 2004).

A 50mer-based oligonucleotide microarray was developed in our laboratory based on known genes and pathways involved in: biodegradation, metal resistance and reduction, denitrification, nitrification, nitrogen fixation, methane oxidation, methanogenesis, carbon polymer decomposition, and sulfate reduction. Rhee et al. (2004) have demonstrated the applicability of this type of array design in studies of degradation of naphthalene from both enrichments and soil microcosms. In this study it was demonstrated using both unamplified DNA and mRNA as targets for probes that different sets of organisms were primarily responsible for naphthalene

degradation in each system. While the naphthalene-degrading genes from *Rhodococcus*-type microorganisms were found to be dominant in enrichments, the genes involved in naphthalene degradation from Gram-negative microorganisms, such as *Ralstonia*, *Comamonas*, *Burkholderia*, were most abundant in the soil microcosms (Fig. 9.2). Although naphthalene degradation is widely known and studied primarily in *Pseudomonas*, genes from these organisms were not detected in either system. Comparatively little is known about the organisms and pathways found to dominate this process in both systems. To verify these results four genes detected in these hybridisations were also amplified and quantified using real-time PCR and results showed good correlation between the two methods (Fig. 9.1).

9.4.3 Community Genome Arrays (CGAs)

The CGA is conceptually analogous to membrane-based reverse sample genome probing (RSGP; Greene and Voordouw 2003). In contrast to RSGP, CGAs use a non-porous surface, a microarray, for fluorescence-based detection. This development enables the utility of RSGP to be scaled up in the number of hybridisations that can be achieved simultaneously allowing high-throughput analyses. These arrays have been used to describe a microbial community in terms of its cultivable component or screen isolates for identification (Cho and Tiedje 2001; Wu et al. 2004). Because of the ease of construction and greater sensitivity, such arrays may be particularly useful for monitoring the abundance of known species that are important in a given environment. Like RSGP, the main disadvantage of the CGAs has been that only the cultured components of a community could be monitored, because the construction required the availability of large amounts of high-molecular-weight genomic DNA from individual pure sources. However, CGA-based hybridisation itself does not require culturing and, with the recent advances in environmental genomics, high-molecular-weight DNA from uncultivated microorganisms can also be accessed through bacterial artificial chromosome (BAC)-based or fosmid-based cloning approaches (e.g. metagenomes). BAC or fosmid clones could then be used to fabricate CGAs, thus allowing the further investigation of uncultivated components of a complex microbial community. Additionally, these types of arrays could potentially be very useful for the screening of metagenomes for specific genes of interest. For example, preselected 16S rDNA targets or functional gene targets involved in C cycling, N cycling, etc., could be hybridised against arrayed environmental BAC or fosmid clones to better identify those clones of interest for further study, such as high-throughput genome-sequencing efforts of inserts.


TFD-NP
IDT
TFD
PCT18
2398777: catechol 1,2-dioxygenase
11323312: dibenzothiophene desulfurisation enzyme (DszD)
21322689: mercuric reductase
11991169: 4-chlorobenzoate CoA ligase
4584854: 4-chlorobenzoate CoA ligase
5824143: alkane-1 monooxygenase
18369658: benzoyl CoA reductase subunit
11967269: 3-oxoadipate enol-lactone hydrolase
14335415: dibenzothiophene desulfurization enzyme (dszA)
1841362: aniline dioxygenase
4827073: cis-naphthalene dihydrodiol dehydrogenase
3641341: lactone-specific esterase
2935025: protocatechuate dioxygenase
3402306: catechol 2,3-dioxygenase
20373111: meta cleavage compound hydrolase (dioxin)
14585958: protocatechuate 3,4-dioxygenase
21115304: cation efflux protein
16943679: cyclopentanone 1,2-monooxygenase
2828018: naphthalene 1,2 dioxygenase
1477923: dinitrotoluene dioxygenase
4220429: aldolase
3337416: salicylaldehyde dehydrogenase
4220434: fumarylpyruvate hydrolase
4234998: naphthalene dioxygenase
18643025: nitrobenzene dioxygenase
1773277: nitrotoluene dioxygenase
4220432: glutathione S-transferase
3894249: phenol hydroxylase
18307552: naphthalene dioxygenase
4220435: maleylpyruvate isomerase
4234963: naphthalene dioxygenase
2828016: salicylate 5-hydroxylase
8118287: cis-naphthalene dihydrodiol dehydrogenase
4220433: gentisate 1,2-dioxygenase
17942397: dinitrotoluene dioxygenase
2828015: salicylate 5-hydroxylase
2463460: 4-hydroxybenzoyl-CoA reductase
12667043: 3-hydroxybenzoate CoA ligase
1040685: benzoate-CoA ligase
12655805: benzoyl-CoA oxygenase
2190573: cyclohex-1-ene-1-carboxylate CoA ligase
2599295: 2,4-dichlorophenol hydroxylase
21322691: mercuric reductase
18150574: naphthalene-inducible monooxygenase
4160661: protocatechuate 3,4-dioxygenase
4096595: protocatechuate 3,4-dioxygenase
3892461: catechol 2,3-dioxygenase
151385: naphthalene dioxygenase
151455: phenol hydroxylase
22252965: naphthalene dehydrogenase
3402328: catechol 2,3-dioxygenase
7619822: phenanthrene dioxygenase (Fd)
2429345: tellurite resistance protein
3059185: 2,3-dihydroxybiphenyl 1,2-dioxygenase
4584855: 4-chlorobenzoyl CoA dehalogenase
13516851: 4-chlorobenzoate-CoA ligase

◄ **Fig. 9.2.** Cluster analysis of naphthalene-enriched (TFD-NP) and control DNA microarray hybridisations using a 50mer oligonucleotide FGA (Rhee et al. 2004). Genes involved in naphthalene biodegradation show significantly greater average hybridisation signals (as indicated by more intense red coloration) in naphthalene-enriched soils. Cluster analyses allow for straight forward visualisation and analysis of differences between microarray hybridisations

Wu et al. (2004) recently tested a multispecies and strain CGA using mixtures of known amounts of DNA or known number of cells from 16 different species. This CGA was used to examine differences in microbial community composition in soil, river, and marine sediments. Consistent with expectations, principal components analysis of the microarray results revealed samples from within each habitat to contain communities more similar to one another than between habitat types (Fig. 9.3). The microarray data also revealed considerable differences for some abundant microbial populations among these samples. For instance, *Marinobacter* species similar to the strains D3-15, D5-10, and E1-7, and *Shewanella* species similar to *S. algae* and *S. oneidensis*, were abundant in the top layer of a marine sediment sample; however, they were not observed in microarray hybridisations of soil and river sediments. Similarly, bacteria similar to the *Pseudomonas* sp. G179, a typical *nir*K-containing soil denitrifier, were abundant in soil samples but not in the river and marine sediment samples. Such results suggest that these arrays may be especially useful for monitoring the abundance of microbial targets of known interest in a given environment.

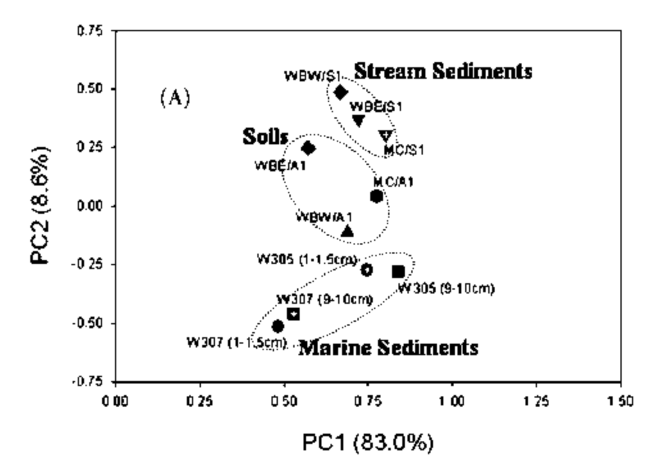


Fig. 9.3. Principal components analysis (PCA) showing the ability of CGAs to distinguish among environmental microbial DNA samples of different origins (Wu et al. 2004). PCA allows for systematic reduction and visualisation of patterns within the large and complex volumes of data generated by microarray analysis

9.5 Conclusions and Future Perspectives

Several challenges still remain for environmental microarrays to reach their full potential. Firstly, inherent experimental variation is a critical issue in microarray hybridisation and novel experimental designs and strategies for evaluating such variation are needed for improving the accuracy of microarray-based studies. Standardisation procedures and technologies need to be developed so that microarray results can be easily compared across laboratories and microarray platforms. Second, microarray hybridisation is still not sensitive enough for some environmental studies when the amount of recoverable biomass is very low and community complexity is very high. Novel methods for increasing detection sensitivity are urgently needed. Third, the quantity of data generated by microarray-based studies of environmental samples will likely be enormous, but rapid processing and mining of hybridisation data still remain difficult endeavours. Finally, although microarrays may provide a rapid means of characterising a microbial community once they are constructed, preparing high-quality samples and probes suitable for microarray analysis appears to be the bottleneck. Automation and improvements in sample processing and probe design are necessary for alleviating this bottleneck.

Despite these remaining issues, the usefulness of this technology is rapidly improving. Once arrays are fully developed, they will be very useful for microbial ecology studies. With fully developed and comprehensive arrays it will become possible to assess simultaneously in a single assay all or most of the constituents of a complex natural community, which could allow us to begin to build a comprehensive view of microbial community dynamics. Although some of the studies reviewed here have shown that microarrays hold promise as valuable tools for analysing environmental samples, the specificity, sensitivity, and quantitative capabilities of microarray technology for environmental applications require close attention and further refinement. Additionally, studies have yet to take full advantage of the high-throughput capabilities of microarrays. More rigorous and systematic assessment, development and applications are needed to realise the full potential of microarrays for microbial ecology studies.

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10 Stable Isotope Probing: A Critique of Its Role in Linking Phylogeny and Function

Mike Manefield, Robert I. Griffiths, Mark J. Bailey, Andrew S. Whiteley

10.1 Introduction

Microbes are responsible for an enormous array of the biochemical transformations in the Earth's biogeochemical cycles and are capable of mineralising almost all chemicals derived from human industry. Whilst these assertions are in no doubt, microbial ecologists have had limited success in attributing biogeochemical and biodegradative transformations to specific microbial taxa in the environment (Gray and Head 2001). This can be attributed to the difficulties associated with observing microscopic processes and our inability to reconstruct all but the simplest of microbial communities in the laboratory. However, in recent times, novel methodological approaches promise to shed light on the relationship between microbial phylogeny and certain community functions in situ (Murrel and Radajewski 2000; Boschker and Middelburg 2002; Radajewski et al. 2003; Wellington et al. 2003). This chapter summarises the lead role a novel methodological approach known as stable isotope probing (SIP) is playing in attributing the assimilation of specific elements from certain compounds to particular microorganisms within complex microbial communities.

Many of the important biogeochemical and biodegradative processes mediated by microbes take place in soil environments (Conrad 1996; Kozdroj and Van Elsas 2001). Owing to their complex and heterogeneous nature, soil environments support the highest microbial species richness per unit of space of any environment known (Torsvik et al. 1990). It follows that the diversity of chemical transformation occurring in soils is also rich; therefore, attributing processes of interest to specific microbial taxa in soil environments is never likely to be a menial task. Despite this, it is clear that through the rigorous implementation of SIP protocols, microbial ecologists are making headway in this endeavour.

SIP involves tracking stable isotope atoms from particular substrates into components of microbial cells that provide phylogenetic information, often

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referred to as biomarkers. The most common biomarkers are nucleic and fatty acids. By aligning the incorporation of an isotopic label with a specific phylogenetic group it is possible to attribute the assimilation of a substrate to certain microbes. Because SIP is founded on assimilatory processes, its utility is restricted to chemical transformations that microbes perform, at least in part, for anabolic purposes. Non-assimilatory chemical transformations, of which there are many relevant to ecology, biogeochemistry and biodegradation, fall outside the applicability of SIP. SIP approaches can theoretically be applied to trace the assimilation of any element that has a stable isotope. Almost all of them do, including most of the abundant elements in biological materials (C, N, O, H, and S). In practice the application of SIP has almost exclusively been restricted to the use of ^{13}C.

SIP methodologies vary in the use of biomarker and the means by which biomarkers are analysed both for isotopic and phylogenetic content (summarised in Fig. 10.1). Whilst not all of the published methodologies have been tried and tested in soil environments they each have potential relevance and are therefore worthy of review here. The remainder of this

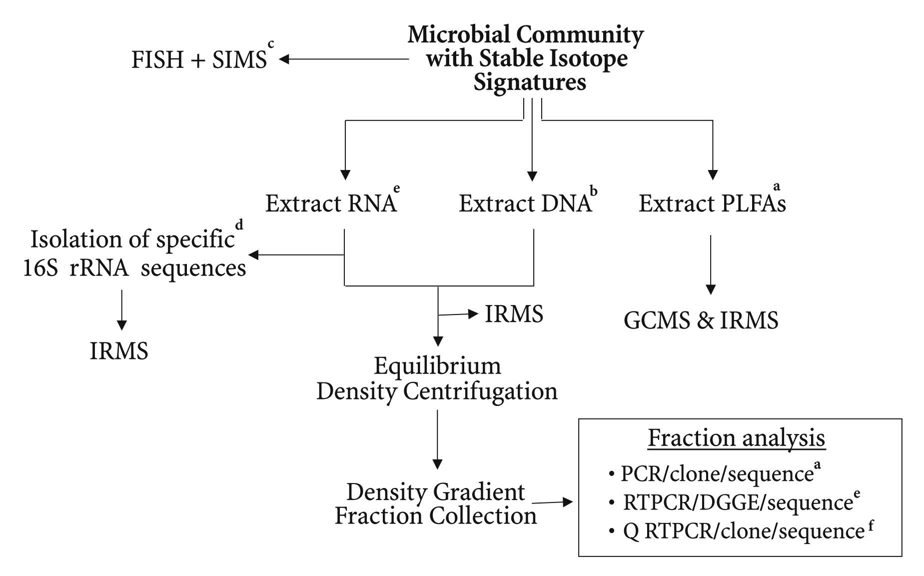


Fig. 10.1. Summary of stable isotope probing (SIP) approaches to link function with phylogeny. Approaches pioneered by *a* Boschker et al. 1998; *b* Radajewski et al. 2000; *c* Orphan et al. 2001; *d* MacGregor et al. 2002; *e* Manefield et al. 2002b; and *f* Lueders et al. 2004a. Equilibrium density centrifugation based SIP methods require pulse enrichments of stable isotope signatures. Refer to text for details. *GCMS* Gas chromatography/mass spectrometry; *IRMS* isotope ratio mass spectrometry; *Q RTPCR* quantitative reverse transcription polymerase chain reaction; *FISH* fluorescence in situ hybridization; *SIMS* secondary ion mass spectrometry; *PLFA* polar lipid derived fatty acid

chapter discusses each in turn with emphasis on practical limitations and the relative contributions each has made to date in linking microbial community functions with phylogeny in the soil environment.

10.2 Polar Lipid Derived Fatty Acid Based Stable Isotope Probing (PLFA-SIP)

Boschker et al. (1998) published the first stable isotope probing investigation, which identified microorganisms responsible for the assimilation of C from the greenhouse gas methane in a freshwater sediment environment. Stable isotope labelled methane ($^{13}CH_4$) was pulsed into the microbial community occupying the sediment resulting in the labelling of polar lipid derived fatty acids (PLFAs) from methane-assimilating organisms. Subsequently, PLFAs were extracted, separated and analysed for ^{13}C enrichment by isotope ratio mass spectrometry (IRMS). Because specific phylogenetic groups produce signature PLFA profiles the stable isotope enrichment of certain PLFAs revealed which organisms were dominating the use of methane as a C source. The labelled PLFAs generated throughout the duration of the pulse were only produced by species belonging to the *Methylobacter* and *Methylomicrobium* genera, thereby unambiguously linking these organisms with methane oxidation in this environment. In the same study, Boschker et al. (1998) identified the sulphate reducing microorganisms responsible for acetate oxidation in estuarine and brackish sediments. PLFA-SIP has also been used to identify the microorganisms responsible for methane oxidation in temperate forest soils (Bull et al. 2000) and marine sediments (Hinrichs et al. 1999) and propionate consumption in anoxic brackish sediments (Boschker et al. 2001). Finally, a number of authors have traced complex substrates such as ^{13}C-labelled plant litter or microalgae into PLFAs in order to investigate the fate of decomposing photosynthetic organisms (Sun 2000; Malosso et al. 2004).

Hanson et al. (1999) were the first to use PLFA-SIP to link the degradation of a xenobiotic to specific microbial community members. Yolo silt loam was pulsed with ^{13}C-labelled toluene and PLFAs were analysed for ^{13}C enrichment. This study attributed toluene degradation to genera within the order *Actinomycetales*. The majority of research attention for toluene degradation has been absorbed by genera within the order *Pseudomonadales*. In this case SIP offered a clear rationale to turn some of this attention to the *Actinomycetales*, and may eventually result in more successful attempts to augment natural toluene remediation. Pelz et al. (2001) performed a similar investigation to identify sulfate-reducing, toluene-degrading microorganisms in the sediment of a petroleum hydrocarbon contaminated aquifer.

Johnsen et al. (2002) traced ^{13}C-labelled phenanthrene into the PLFAs of various phenanthrene-degrading taxa in soils unpolluted, lightly polluted and heavily polluted with polyaromatic hydrocarbons. Alexandrino et al. (2001) used PLFA-SIP to identify styrene-degrading organisms from biofilters used for the treatment of styrene-containing waste gases. Uniquely, they applied a ^{2}H- rather than ^{13}C-labelled substrate in their investigation. To date ^{2}H has not been widely used in SIP applications, and there are no published examples of its application in the soil environment.

The most striking advantages of using PLFAs as biomarkers in SIP is the rapidity with which PLFAs become detectably labelled and the quantitative information generated from the analysis of PLFAs with IRMS. The primary limitations of PLFA-based SIP are that the phylogenetic resolution offered by this biomarker is inferior to that offered by nucleic acid (NA)-based biomarkers and that it requires signature PLFAs to have been identified from close culturable relatives. A number of researchers also regard the extraction of PLFAs from soils as a labour-intensive process. Despite these limitations PLFA-based SIP has implicated more soil microbes in environmental processes of interest than any other form of SIP.

10.3 DNA- and RNA-Based Stable Isotope Probing (NA-SIP)

In comparison to PLFAs as a biomarker, polymerase chain reaction (PCR)-based sequence analyses of 16S rDNA genes or rRNA itself offers high-resolution, culture-independent phylogenetic information (Olsen et al. 1986). Radajewski et al. (2000) enhanced the phylogenetic resolution of SIP by demonstrating that stable isotope labelled DNA could be isolated from mixed microbial communities based on the increase in buoyant density associated with isotopic enrichment. Equilibrium density centrifugation in CsCl gradients was used to separate ‘heavy’ (labelled) from ‘natural’ (unlabelled) DNA and 16S rRNA gene clone libraries constructed from ‘heavy’ DNA were sequenced to obtain the identity of organisms assimilating the ^{13}C-labelled substrate used in the study (Fig. 10.2).

This technique was used to attribute methane and methanol utilisation in oak forest soil microcosms to moderately acidophilic methylotroph populations (Radajewski et al. 2000, 2002). Radajewski et al. (2002) went on to amplify functional genes involved in the oxidation of one-carbon compounds from high density gradient positions. Along with implicating methanol dehydrogenase and methane monooxygenase genes in methanol and methane assimilation, this study found that species encoding ammonia monooxygenase had assimilated ^{13}C. It was proposed that ammonia-oxidising species had assimilated ^{13}C second hand through the $^{13}CO_2$ generated by the

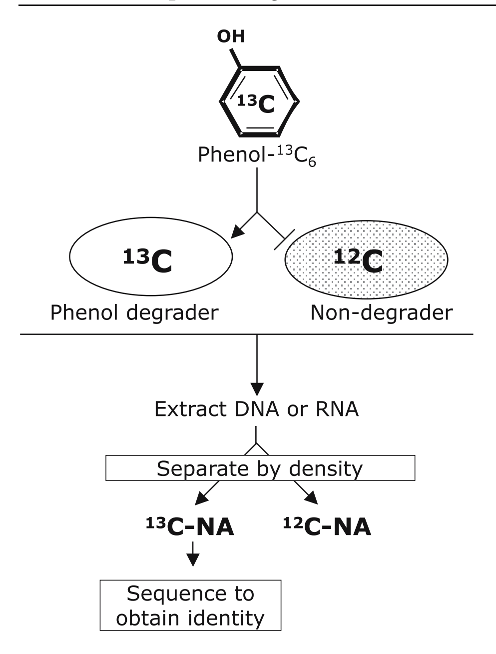


Fig. 10.2. An example of nucleic acid (NA)-based stable isotope probing. A ^{13}C-labelled substrate (e.g. phenol) is pulsed into a mixed microbial community composed of phenol-degrading and non-phenol-degrading organisms. After the pulse is consumed, total community DNA or RNA is extracted and separated by equilibrium density centrifugation. NA sequences are then obtained from the ^{13}C-labelled fraction thereby revealing the identity of organisms acquiring carbon from the chosen substrate (phenol)

methylotrophs. This raises the interesting challenge posed by metabolic crosstalk. Whilst SIP has the potential to dissect microbial food webs, to date the possibility of secondary feeding on breakdown products of the primary substrate has emerged as a frustrating caveat researchers cannot ignore.

SIP with DNA has also been used to identify active methanotrophs in peat soil (Morris et al. 2002) and ammonia-oxidising microbes in freshwater sediments (Whitby et al. 2001). One of the primary limitations of NA-SIP is the requirement for a large isotopic enrichment in the biomarker. This, in conjunction with concerns over the presence of unlabelled substrates native to the system that will compete for assimilation, has led researchers to apply artificially high concentrations of labelled substrates into soil microcosms for extended periods of time. The in situ relevance of doing so has been questioned (Jeon et al. 2003; Padmanabhan et al. 2003). Jeon et al. (2003) pulsed ^{13}C-labelled naphthalene at realistic concentrations into a contaminated sediment to identify naphthalene degraders in situ. Further, Jeon et al. (2003) were able to isolate a β-proteobacterium, implicated in

naphthalene mineralisation by DNA-SIP, and demonstrate that it encodes a naphthalene dioxygenase present at the field site. It is pertinent to mention at this point that, owing to the heterogeneous nature of soil environments, the diversity between different soil environments (including the ability to adsorb different compounds) and the diversity in solubility and volatility of the many substrates relevant to SIP in soil environments, that careful planning of the delivery of a pulse is crucial to the generation of meaningful results.

Despite the successful application of DNA-SIP to naphthalene degradation in the field, the requirement for cell replication during pulse labelling, which is notoriously slow in soil environments, will always be a limitation to the isotopic enrichment of DNA. Whilst extending the duration of a pulse can alleviate this limitation it also exacerbates the issue of metabolic crosstalk. To resolve this dilemma, 16S rRNA-based SIP methodologies have been developed (Manefield et al. 2002a,b). Because RNA is turned over in bacteria independently of replication, rates of RNA synthesis are always higher than for DNA. For this reason RNA will be labelled more rapidly, making it a more responsive biomarker with a high degree of phylogenetic resolution.

The applicability of RNA-SIP was demonstrated with the identification of bacterial species responsible for phenol degradation in the activated sludge community of an industrial waste water treatment plant (Manefield et al. 2002b). Manefield et al. (2002b) have additionally highlighted the importance of fractionating equilibrium density gradients and monitoring increases in NA density over time as a substrate pulse is assimilated. The utility of the technique in the soil environment has been demonstrated by Lueders et al. (2004b) who identified methylotrophs responsible for methanol assimilation in a rice field soil and implicated certain fungi and flagellates in grazing on these methylotrophs. They have gone on to use RNA-SIP to identify the bacteria and archaea responsible for syntrophic propionate degradation in flooded soil, including representatives of uncultured phylogenetic groups (Lueders et al. 2004c). Lueders et al. (2004a) have introduced the quantification of transcripts or genes in gradient fractions with real-time PCR. Even though the use of RNA as a biomarker and the rigorous quantitative examination of gradient profiles have enhanced the sensitivity of NA-SIP, there remain certain limitations to its application in soil environments. An obvious hurdle is the ability to extract clean and intact DNA or RNA from the soil environment under investigation. A less intuitive limitation is exemplified by the following contemplation of unpublished data from our laboratory.

Given that the soil microbiota plays a large role in regulating the concentrations of greenhouse gases in the atmosphere, many researchers have recognised the value of attributing particular processes in soil to specific

taxonomic groups present therein. For example, there is a need to identify the microbial taxa dominating the acquisition of carbon from plant root exudates. To address this question, we pulsed labelled grassland turfs with $^{13}CO_2$ to generate and deliver ^{13}C-labelled root exudates to the soil community (Ostle et al. 2003), and then analysed the 16S rRNA from root-associated soil by IRMS and equilibrium density centrifugation (Griffiths 2003). Whilst the IRMS analysis revealed that the RNA was enriched with ^{13}C, the degree of labelling was too low to allow conclusive results to be drawn from density centrifugation experiments. A PLFA-SIP analysis of soil samples derived from a $^{13}CO_2$ plant pulse was able to attribute assimilation of root exudates at low phylogenetic resolution to Gram-negative bacteria and fungi (Treonis et al. 2004).

This raises an important issue related to any SIP methodology that is dependent on the degree of labelling. If the labelled substrate of choice will ultimately be consumed by a rich diversity of organisms then the degree of labelling in any given taxa will be low, making separation by density problematic. Conversely, if the substrate of choice is consumed by a small number of taxa, then the degree of labelling in those taxa will be high, facilitating isolation by density. The use of $^{13}CO_2$ to label soil microbes via root exudates is particularly problematic both because the initial substrate is translated into a large range of organic compounds and the microbial diversity in the soil is extremely rich. Further, the physiological state of genetically identical cells in the soil environment can be very different at any one time. Ultimately, attributing the assimilation of root exudates to specific taxa by NA-based SIP will involve pulsing labelled exudates directly into root-associated soil. There are experimental systems available to mimic the delivery of root exudates into soil (Falchini et al. 2003).

10.4 Alternative Stable Isotope Based Approaches

Two additional and distinct stable isotope based approaches have been developed to unveil the relationship between microbial community functions and diversity. Orphan et al. (2001, 2002) have published investigations in which individual cells or mixed aggregates of cells in methane-consuming communities were identified by fluorescence in situ hybridisation (FISH) and subsequently analysed for ^{13}C content by secondary ion mass spectrometry (SIMS). The natural abundance of ^{13}C in methane is conveniently low, enabling Orphan et al. (2001) to associate cells harbouring depleted ^{13}C signatures with methane consumption. In this way, the involvement of specific Archael groups in the consumption of oceanic methane reservoirs in marine sediments has been demonstrated.

A related approach involves isolating the small-subunit (SSU) rRNA of particular phylogenetic groups from total community RNA and determining stable isotope levels therein by IRMS. Specific rRNA molecules can be captured by the hybridisation of biotin-labelled probes to target sequences followed by retrieval of hybridised targets using magnetic beads. MacGregor et al. (2002) demonstrated the potential utility of this approach using laboratory grown cultures. Again, natural differences in the stable isotope composition of substrates could be exploited to trace their assimilation into phylogenetic groups of interest. The primary technical challenge associated with this method is the isolation of sufficient quantities of rRNA for isotopic characterisation. This approach awaits application in the field.

The primary advantage of the Orphan and the Macgregor approaches is that there is no requirement for high degrees of labelling for the method to be informative. As demonstrated, these methods can be applied to scrutinise natural stable isotope signatures. Further, the method can also be used in conjunction with an enrichment pulse. The disadvantage of these approaches lies in the fact that it is dependent on sets of predetermined oligonucleotides that must be hybridised to sequences of interest. This necessarily transforms the question being asked from 'which taxa assimilate the substrate?' to 'does a specified taxa assimilate the substrate?', thereby pre-determining the analysis to some degree.

10.5 Radioactive Isotope Based Approaches

The use of stable isotopes to trace the assimilation of elements from substrates of interest by microbial taxa in soil environments has, in some ways, overshadowed exciting developments of radioactive isotope based methods aimed at achieving the same alignment of function with phylogeny. By combining FISH with microautoradiography (MAR), Lee et al. (1999) demonstrated that the assimilation of ^{14}C-labelled acetate, butyrate and bicarbonate could be attributed to various taxa in activated sludge samples. Whilst the number of published studies making use of MAR-FISH continues to accrue, it remains to be applied to soil environments. Technical aspects of the methodology in the context of soil matrices may ultimately make it too difficult to do so.

An approach that has more potential in its application to soil environments makes use of 16S rRNA microarrays (Adamczyk et al. 2003). After pulse labelling activated sludge samples with ^{14}C-labelled bicarbonate, Adamczyk et al. (2003) hybridised community 16S rRNA samples to a microarray which was then scrutinised for the location of the radioactive isotope in order to identify the autotrophic members of the community.

Despite the obvious utility of these radioactive isotope based techniques, their potential to link community structure to function in field experiments is nullified by the associated and valid health and safety concerns. As evidenced by the rapid development and application of alternative stable isotope based approaches, many researchers prefer to avoid working with radioactivity.

10.6
Notes on Isotopic Enrichments

Most of the studies described above have involved enriching isotopic signatures in biomarkers of microbes performing assimilatory functions. A small number of them have exploited natural isotopic signatures derived from processes that fractionate against rare isotopes. The presence of natural isotope signatures are a fortuitous phenomenon inherent to a limited number of biochemical transformations, not only because there is a ready-made signature to observe, but also because it avoids two important considerations that researchers performing enrichment pulses must take into account. The first is that the presence of a label may cause a substrate to be discriminated against. Our experience with phenol and SIP suggests fractionation does not occur on a discernable level in the face of an enrichment pulse, but this does not rule out the possibility that fractionation may manifest itself in other situations. The use of natural isotopic signatures also avoids the expense of acquiring labelled substrates. In many cases the cost of having a particular fully labelled substrate custom-synthesised is too high to justify the use of a stable isotope probing approach.

10.7
Conclusions

Herein we have described the benefits being derived from the development of SIP alongside related caveats in the context of linking microbial phylogeny and function in soil environments. It is clear from the increasing frequency of publications in which SIP plays a role that it has great utility in its various guises, even in soil environments. We believe that with considered application SIP has the potential to confirm or discover the identity of microbes responsible for a large range of processes of interest to humans both in fundamental ecology and bioremediation. Despite this, we would still argue that there is room for methodological improvement. Ideally, we require a SIP methodology that allows the quantification of label incorporation in conjunction with sequencing based phylogenetic

resolution. Further, it is important to emphasise that SIP can only penetrate the relationship between microbial community composition and function when the function of interest involves anabolic activity. There are countless important activities partaken by microbes that do not involve substrate assimilation. Despite the promises of SIP, microbial ecologists have at hand the challenge of developing even more ingenious means of unravelling the relationship between specific taxa and non-assimilatory functions.

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11 Gene Detection, Expression and Related Enzyme Activity in Soil

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11.1 Introduction

Molecular techniques have been used to detect functional genes present within soil bacterial communities, to establish the potential for key microbial functions in soil such as plant growth promotion and disease suppression, nitrification, nitrogen fixation, denitrification and decomposition. The use of reverse transcription polymerase chain reaction (RT-PCR) has enabled estimation of active functional groups, which, with other techniques such as real-time PCR, has allowed major developments in our understanding of how soil treatments and the environment affect soil microorganisms. There have been attempts to unravel the interrelationship between functional and taxonomic diversity and activity (ammonia-oxidisers, Okano et al. 2004; 2,4-dichlorophenoxyacetic acid degraders, Kamagata et al. 1997; streptomycin-producers, Huddleston et al. 1997; chitinolytic bacteria, Metcalfe et al. 2003) but the extent of functional redundancy in soil still needs to be explored for key processes involved in decomposition and mineral recycling. It will be a considerable challenge to relate function to community structure given recent findings concerning the need for representative sampling to estimate habitat diversity (Homer-Devine et al. 2004) and the predicted extensive diversity within soil microbial populations (Torsvik and Ovreas 2002). The relationship between microbial diversity and soil functions has been reviewed (Nannipieri et al. 2003) with consideration of the relationship between functioning of the soil in terms of decomposition, resilience and general activity as measured by soil respiration. This holistic approach has not provided any clear relationship between microbial diversity and activity in soil. Soil is a heterogeneous habitat providing a very large surface area for colonisation; the extent of the soil surface colonised by bacteria appears to be very small (< 10%; Clewlow et al. 1990). This adds to the sampling problem where representative sampling may require intensive analysis of large sample sets in order to estimate

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prokaryotic diversity. Thus a targeted approach may well be required to focus on specific aspects of microbial activity in soil, as discussed by Curtis and Sloan (2004), who emphasised the problems of sampling microbial population diversity. A range of functional gene targets is available for detection of key microbial activities in soil in addition to taxonomic signature sequences linking structure to function such as 16S rRNA targets for nitrifiers. Phylogenetic analysis of functional gene sequences can give an indication of the host background if there is conservation within the target sequences. However, many activities of soil bacteria may be adaptations to the soil environment and subject to horizontal gene transfer (HGT) thus obscuring phylogenies. Advances in metagenomics of the soil microbial population may assist in resolving the link between structure and function by analysis of large DNA fragments (HMW DNA) linking highly conserved genes, such as 16S rRNA, with adaptive features, e.g. extracellular enzyme secretion or energy generation pathways.

Soil samples can be screened for the presence of specific genes allowing the analysis of many environmental samples, although the level of sensitivity will vary with soil type. Methods have been developed to increase the number of genes targeted such as the use of DNA microarrays as 'genosensors' for the detection of specific microorganisms in environmental samples (for more details, see Chap. 9). The major advantage of this technique is that many sequences can be placed on one chip and the sample can be simultaneously analysed for the presence of many diverse target groups.

The highly complex trophic interactions that occur amongst microorganisms present a challenge to unravel the relationship between metabolic and genotypic diversity (Zak et al. 1994). Microorganisms have a key role in the environment, playing a major part in the decomposition of polymers and degradation of environmental contaminants (Lamar and Dietrich 1990; Aelion and Bradley 1991). One of the better-understood processes is the impact of bacteria on nitrogen cycling in soil and the role of key genotypes in nitrification (Prosser and Embley 2002).

Methods for studying specific activities, such as an enzyme activity or the ability to degrade a toxic compound, give little information on the identity of key bacteria. Conversely, the presence of specific genes giving taxonomic information can only indicate identity of the host population, but can never confirm its role unless metagenomics is used to link taxonomic and functional gene targets. However, the analysis of mRNA extracted from soil can provide useful data on activity of certain genotypes. Proteomics also promise to contribute to our understanding in this field (for more details, see Chaps. 4 and 5). Despite the complexity of the soil microbiota there has been significant progress in the analysis of functional genes in relation to traditional measurements of soil sample activity. In addition, surveys of target gene prevalence and diversity have provided indications of im-

pacts of agricultural practice on soil microbial communities such as the association between ergotrophic use of antibiotics and the prevalence of antibiotic resistance genes in manured soils and farm microflora (Seveno et al. 2002). Molecular detection of genes involved in degradation of pollutants in soil has provided data on prevalence and diversity of key enzymes involved, such as the in situ detection of *mmoX* genes coding for part of the soluble methane monoxygenase enzyme complex involved in the oxidation of trichloroethylene (Shigematsu et al. 1999).

This review aims to summarise selected recent developments in attempts to define microbial populations responsible for a defined activity, enzyme or process in soil by targeting specific genes. The direct molecular analysis of gene presence in soil via analysis of total community DNA (TC-DNA) and measurement of activity by targeting mRNA can be illustrated by a number of case studies. Examples have been selected on the basis of relevance to soil processes and attempts to evaluate the impacts of agricultural practice, pollution and climate on soil. Selected studies have attempted to quantify gene copies, which can be done by quantitative PCR (QPCR) by measuring the concentration of PCR product using densitometry, competitive PCR, and real-time PCR. Quantity of target sequences in TC-DNA extracts, whatever method of estimation, must be compared to a calibration curve, either as enumerated cells in an extract of chromosomal or plasmid origin or by addition of known number of targets to a TC-DNA extract that does not contain the target gene of interest. Few studies have attempted to determine if QPCR with chromosomal or plasmid DNA is directly equivalent to the same reaction using TC-DNA. It is preferable to use seeded TC-DNA for calibration. Quantifying molecular targets in TC-DNA will only serve to estimate the potential activity for a given specific population, even when derived from mRNA. The latter is more likely to indicate actual activity in a soil extract but transcripts can give rise to inactive enzymes which require further processing. Thus it is important that any gene being used as a target to identify a potential functionally active population will have been well studied in vitro and molecular mechanisms of regulation understood. All molecular data should be used in conjunction with studies of enzyme activity and measurements of general microbial populations in a soil such as ATP, AEC (adenylate energy charge ratio determines the growth state of natural microbial communities and the effect of environmental changes on them), biomass and cell counts.

11.2 Molecular Detection of Functional Genes in Soil

11.2.1 Introduction

A wide range of genes have served as targets for studies of microbial activity in soil, but some trends emerge for the study of a range of microbially driven processes. These include antagonism and biological control and the ability of the soil microbiota to respond to various impacts such as pollution and agricultural practice. The latter involves genes expressed in bioremediation studies, heavy metal and antibiotic resistance. Further important microbial activities relate directly to soil fertility and the cycling of key nutrients such as the genes implicated in the carbon and nitrogen cycles where specific activities can be attributed to key genes such as those involved in nitrification or biodegradation of recalcitrant polymers. Detection of functional gene signatures of pathogenic microorganisms in the soil environment represents another important issue, the significance of which has been acknowledged during recent years.

Extraction of Microbial Biomass from Soil

Detection of genes in TC-DNA extracted from soil poses technical problems associated with the difficulty in extracting DNA of sufficient quality for use with PCR and other methods. Co-isolation of substances which inhibit PCR amplification occurs, particularly humic acids, and a variety of extraction protocols and purification methods have been developed in an effort to provide PCR quality TC-DNA (Frostegård et al. 1999; Krsek and Wellington 1999; Griffiths et al. 2000; Courtois et al. 2001; Sessitsch et al. 2002). Nucleic acid extraction from soil is discussed in detail in Chap. 3.

A range of methods is available to concentrate soil microbes, and remove the soil particles, which may inhibit down-stream applications such as PCR. Where microbial biomass is extracted from large volumes of soil, sensitivity is also increased. Microbial cells extracted directly from soil without cultivation provide an opportunity to examine the physiological state of cells and identify individual genes using fluorescence in situ hybridisation (FISH) or using fluorescence activated cell sorting (FACS), where specific genes are labelled with fluorescent oligonucleotide probes.

Extraction of microbial cells requires physical or chemical dispersion of soil to separate cells from the soil matrix. Cells adhere to soil particles in many ways including electrostatic and hydrogen bonding. Cells are also physically entrapped within the soil aggregates (Stotzky 1986; Robert and Chenu 1992). The type and strength of microbial attachment will depend

on the soil type and the identity and activity of the microbial community. Once the soil matrix is dispersed a further concentration step is used to separate the biomass fraction from soil debris.

Shaking, blending using a Waring blender or stomacher and ultrasonication are used to physically disperse soil samples. Gram-negative organisms and those sensitive to lysis using aggressive methods such as homogenisation using a Waring blender or ultrasonication may be better suited to dispersal by stomaching or shaking. *Salmonella* spp. were efficiently recovered from soil using a stomacher for dispersion combined with a chemical dispersion and centrifugation (Turpin et al. 1993). It has been demonstrated that ultrasonication may cause more lysis than other methods and this has been avoided by using mild ultrasonication (Hopkins and O'Donnell 1992).

Chemical dispersion using the ion-exchange resin Chelex 100 (reduces the electrostatic attraction between cells and soil particles) has been widely used for extraction of microbial biomass although Tris buffer and sodium hexametaphosphate have also been used (Niepold et al. 1979; Herron and Wellington 1990).

Buoyant density has been used to separate soil particles and microbial cells; this relies on the assumption that most cells are not aggregated and are detached from soil particles. The soil type is important in the success of this method; soils with a high organic content, for example, will have less dense particles and may be impossible to separate from the cells. A number of gradient media have been used but Percoll has been used extensively and allowed the recovery of a clean bacterial fraction for biochemical studies (Bakken 1985). A single step method for purification may be possible with the density material Nycodenz (Bakken and Lindahl 1995). A recent protocol (Berry et al. 2003) blended soil with disruption buffer and then soil homogenate was transferred to an ultracentrifuge tube and Nycodenz pipetted to form a layer below the homogenate. After centrifugation a faint whitish band containing bacterial cells appeared at the interface between the Nycodenz and the aqueous layer. Nycodenz extraction can be used to separate cell fractions of sufficient purity to allow direct flow cytometric analysis (Unge et al. 1999).

Immunomagnetic capture (IMC) has been used to recover cells from environmental samples (Morgan et al. 1991; Fluit et al. 1993). Microscopic magnetic beads (such as those manufactured by Dynal) coated with monoclonal or polyclonal antibodies are used to extract target bacteria. Their major use to date has been in the detection of medically important species in food and water, but methodological modifications for retrieval of salmonellae from soil are currently in development by the authors. The feasibility of this approach is supported by studies conducted by Schmidt et al. (1968) who used fluorescent-antibody techniques to study free living soil bacteria such as rhizobia. Autofluorescence was not a problem but non-specific

absorption restricted observation of rhizobia to microscopic fields free of soil particles. Frederickson et al. (1988) used fluorescent antibodies to enumerate Tn5 mutant bacteria in soil; the fluorescent antibody method tended to overestimate the viable population. The IMC approach provides a useful alternative to the more traditional isolation procedures and does not require the cultivation of the target cells. In addition a highly purified cell extract is produced as the captured magnetic beads can be washed and all soil particles removed. The majority of capture techniques have used a direct IMC approach where either monoclonal antibodies or specific polyclonal antibodies were used to coat magnetic beads; an indirect approach is based on the principle where primary target antigen antibodies conjugated to a secondary antibody are added to the sample followed by the addition of beads with antibodies designed to conjugate with the secondary antibodies. Both direct (Wipat et al. 1994) and indirect methods (Mullins et al. 1995) have been applied to soil extracts; the direct method may be preferable if monoclonal antibodies are used as the polyclonal purified IgG fraction may contain $< 30\%$ specific antibodies. IMC techniques have recovered very pure fractions of specific cells from dispersed soil extracts but the recovery efficiency can vary from 4–90% depending on the type of soil and technique used. Porter et al. (1998) reported isolation of *E. coli* O157 from soil using commercially available anti-*Escherichia coli* O157-labelled magnetic beads.

Magnetic capture systems can also be used to obtain specific genes directly from TC-DNA. Jacobsen (1995) used a magnetic capture-hybridisation PCR technique (MCH-PCR) to eliminate the inhibitory effect of humic acids and other contaminants in PCRs targeting specific soil DNA. A single-stranded DNA probe, which was complementary to an internal part of the target gene, was used to coat magnetic beads. After hybridisation in a suspension of soil DNA, magnetic extraction of the beads separated the hybrid DNA from all other soil DNA and humic acids.

11.2.2 Antibiotic Biosynthesis Genes

The production of antibiotics by bacteria, a vast majority of which were isolated from soil, has prompted much research and speculation into the possible production and role of antimicrobial secondary metabolites in soil (Anukool et al. 2004). Although antibiotics have not been isolated from natural untreated soils, production has been detected in non-sterile, amended and sterile unamended and amended soils when inoculated with producing strains of streptomycetes and fungi (Williams 1982; Wellington et al. 1993). There is evidence to support the occurrence of antibiosis in soil,

e.g. survival of a *Salmonella* sp. in soil was inhibited by the presence of streptomycin-producing *Streptomyces bikiniensis* (Turpin et al. 1992). Evidence for antibiotic production in soil can be achieved by either detection of the active product extracted from soil or by detecting activity of specific promoters associated with antibiotic production using reporter genes such as *lux*. These approaches are also discussed in Sect. 11.3.2.2.

Degenerate PCR primers designed to detect antibiotic biosynthesis genes have been used to study both diversity and distribution of potentially bioactive bacteria in soil. Key enzymes involved in the biosynthesis of certain chemical classes of antibiotic such as the type II polyketide antibiotics have been targeted.

Despite their apparent structural diversity, polyketides share a common mechanism of biosynthesis. The chemical synthesis of polyketides is centred around the reactive groups of the ketone and its adjacent α-methylene carbon (Kramer and Khosla 1996). Therefore, three opportunities exist for structural diversity, the nature of the starter unit, the extender unit and the stereochemistry resulting from their condensation. The carbon backbone of a polyketide results from sequential condensation of short fatty acids, such as acetate, propionate, or butyrate, in a manner resembling fatty acid biosynthesis but catalysed by polyketide synthases (Hopwood 1997). The phylogenetic relationship of amino acid sequences from type II PKS, KSα and KSβ genes was examined by Hopwood (1997) and were found to cluster separately suggesting different functions of these genes. Within these individual clusters the genes encoding polyketide spore pigments were found to be separate from those encoding the antibiotic (and other bioactive) polyketides. This suggests a separate line of descent for these two groups.

A detailed study of polyketide synthase (type II ketosynthase KSα genes) diversity in soil was undertaken by sequence analysis of PCR products derived from use of consensus primers for a conserved region in type II KS genes covering both KSα and KSβ (Morris 2000). The majority of sequences recovered did not match to known genes but were recovered in distinct clades, as shown in Fig. 11.1. The list of known genes given in Table 11.1 was used to design the primers and compare cloned sequences recovered from TC-DNA. This study showed that bacteria with the potential to produce polyketide antibiotic-like metabolites and also pigments were widespread in soil and there was an extensive metabolic diversity not yet explored.

Seow et al. (1997) used PCR primers to amplify KSβ genes from TC-DNA extracted from soil. These products were cloned and two clones sequenced. Both sequences were found to cluster within known type II PKS gene clusters but again did not match any existing genes. These sequences were used to complement mutants for design of novel polyketide antibiotics.

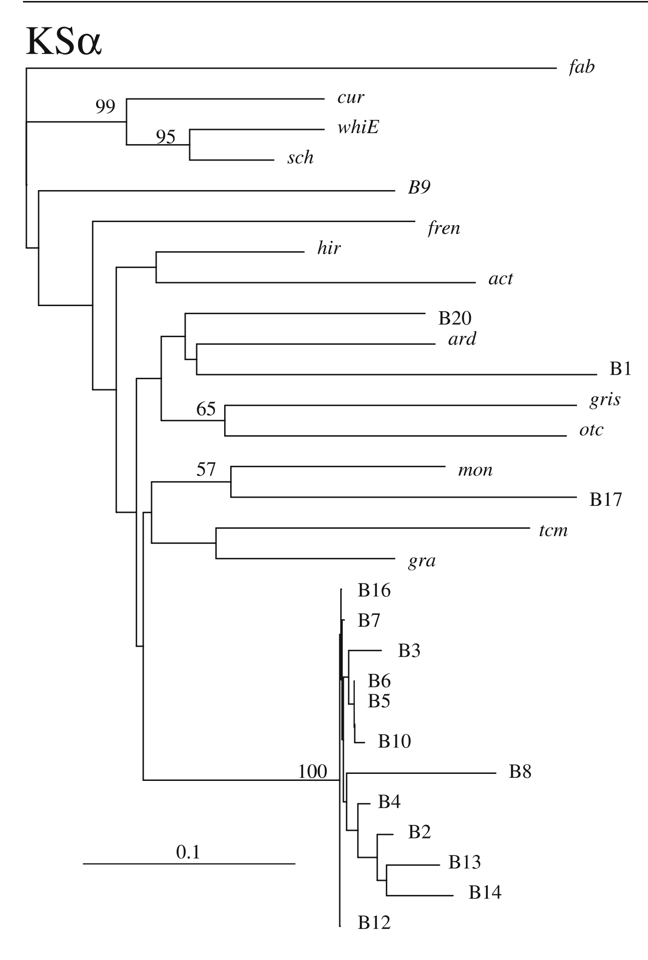


Fig. 11.1. Neighbour-joining tree of partial nucleotide sequences (400 bp) of ketosynthase (KSα) genes obtained from bulk soil (B). Sequences were compared to actinomycete-type strains and the *S. glaucescens* FAS condensing enzyme as an outgroup. For key, see Table 11.1

11.2.3 Detection of Antibiotic and Heavy Metal Resistance Genes

There have been relatively few studies focusing on detection of antibiotic or heavy metal resistance genes in situ, in environments such as soil. Most research has concentrated on detection of resistance genes in clinical and environmental isolates. Resistance genes have been detected in soil using conventional DNA extraction and PCR methods. Chee-Sanford et al. (2001) screened for eight *tet* genes in groundwater associated with swine production facilities. Tetracycline resistance genes were found as far as 250 m

Table 11.1. Cloning and sequencing of aromatic PKS gene clusters from actinomycetes (reproduced from Hopwood 1997)

Host	Polyketide	PKS genes	Cloning strategy
S. coelicolor	actinorhodin	*act*	Complementation
S. rimosus	oxytetracycline	*otc*	Resistance/ complementation
S. glaucescens	tetracenomycin	*tcm*	Complementation
S. violaceoruber	granaticin	*gra*	*act* probe
S. coelicolor	spore pigment	*whiE*	Complementation/*act* probe
S. peucetius	daunorubicin	*dps*	*act*/*tcm* probes
S. cinnamonensis	unknown	*mon*	*act* probe
S. halstedii	spore pigment	*sch*	*act* probe
S. curacoi	spore pigment	*cur*	*act* probe
Sac. hirsuta	unknown	*hir*	*act* probe
S. roseofulvus	frenolicin	*fren*	*act* probe
S. griseus	griseusin	*gris*	*act* probe
S. venezuelae	jadomycin	*jad*	*act* probe
S.spp. C5	daunorubicin	*dau*	*act* probe
Kib. aridum	unknown	*ard*	*act* probe
S. fradiae	urdamycin	*urd*	*tcm*/*act* probes
S. nogalater	nogalamycin	*sno*	*act* probe
S. argillaceus	mithramycin	*mtm*	*act* probe

downstream from waste lagoons, highlighting the danger posed by use of antibiotics in agriculture and the risk of contamination of drinking water with antibiotic resistant bacteria. In a different study a detection limit of 10^2–10^3 copies of the *tet*(M) gene per gram was achieved using a nested PCR method with TC-DNA (Agersø et al. 2004). The gene was detected in farmland soil previously amended with pig slurry containing resistant bacteria; the number of positive samples from farmland soils 1 year after manure treatment was significantly higher than in samples of garden soil not treated with manure.

Exogenous plasmid isolation has been used to detect resistance genes in soil bacteria. This method allows capture of plasmids from the total bacterial fraction of an environmental sample without the necessity to culture the host organism. Smit et al. (1998) investigated mercury resistance plasmids in soil populations using exogenous isolation, and identified plasmids of 10–50 kb carrying resistance to copper, streptomycin and chloramphenicol. These authors amended soil with mercuric chloride and found this to subsequently increase the recovery of resistance plasmids. Plasmids have also been captured from polluted soils and slurrys (Top et al. 1994; Smalla et al. 2000). The former authors identified multiple antibiotic resistance genes from isolated plasmids.

In addition to multi-resistance plasmids antibiotic resistance genes are situated on transposable elements that can associate with other elements such as chromosomes and plasmids. These transposable elements include transposons and integrons, which can be transferred horizontally. Integrons are thought to play an important role in the evolution of antibiotic resistance as they contain mobile gene cassettes which bear a recombination site known as a 59-base element (59-be) that is recognised by the integron-encoded integrase IntI (Hall et al. 1991). Most cassette genes described are antibiotic or quaternary ammonium compound resistance genes. However, recent studies have revealed that the cassette gene pool is far more diverse than previously thought. Stokes et al. (2001) designed PCR primers to conserved regions within the 59-be of gene cassettes, allowing detection of a large number of novel genes. Using these primers, 123 cassette types were recovered from Antarctic and Australian soils and sediments, with very few represented in clone libraries more than once, indicating the large size of the cassette gene pool. Most open reading frames (ORFs) did not match known sequences, again illustrating the diversity of these gene sequences. Further studies revealed a further 41 environmental gene cassettes, giving a total of 164 directly sampled from natural environments by PCR (Holmes et al. 2003). There are several classes of integrons, the most commonly studied being class 1 integrons that are commonly associated with antibiotic-resistant bacteria. Recent studies have detected novel integron classes in soils: Nield et al. (2001) classified three new integron classes and Nemergut et al. (2004) an additional 14 classes, demonstrating the immense variety of these elements present in the environment.

Antibiotic resistance genes have proved useful as molecular markers for studying the antibiotic-producing streptomycetes in soil. Self-resistance genes in antibiotic-producing bacteria are usually clustered with the biosynthesis genes and may indicate potential for antagonism in addition to resistance. The presence of indigenous antibiotic resistance and production genes in soil TC-DNA was investigated and allowed detection of specific genes by PCR amplification. The genes involved in streptomycin production were detected in diverse samples of natural and agricultural Brazilian soils (Huddleston et al. 1997). This indicated the presence of a population of actinomycetes with the potential for antibiotic production, probably in excess of 10^3 colony-forming units (cfu) g^{-1}, which is about the detection limit for DNA from soil samples using PCR. Using a similar approach the distribution of the gene *sttR* involved in self-resistance to streptothricin was studied in a wide range of soils from Europe and sub-tropical regions. DNA sequencing of the PCR products from TC-DNA from two Cuban soils, sugar cane and maize soil, were the only samples to provide PCR products. Since the first round of PCR yielded a small amount of DNA that was not sufficient for DNA sequencing, the PCR products were reamplified with the

same primers before being sequentially purified and sequenced (Watyam 2003; Anukool et al. 2004). The Blast results confirmed that these sequences had homology to streptothricin resistance genes from known streptothricin producers in the databases.

11.2.4 Nutrient Cycle Genes; the Nitrogen Cycle

The nitrogen cycle provides a good example where functional gene microarrays have been developed to monitor dynamics of functional groups (Taroncher-Oldenburg et al. 2003). The arrays rely on hybridisation with oligonucleotides encompassing different variants of the genes involved in nitrogen fixation (*nifH*), the first stage of nitrification involving ammonia oxidation (*amoA*), and denitrification (*nirK* and *nirS*). Nitrification is a key microbial driven process in soil and the oxidation of ammonia to nitrite is attributed to ammonia oxidisers followed by oxidation to nitrate by nitrite oxidisers. Molecular studies of nitrification have concentrated mainly on ammonia oxidisers and 16S rRNA PCR primers were developed for the detection of known members of this functional group (McCaig et al. 1994). The diversity within the α-subunit of the ammonia monooxygenase (*amo*A) has been used to evaluate nitrifying potential in grassland (Webster et al. 2002). Phylogenetic comparison of 16S rRNA genes and *amo*A genes indicated similar evolutionary relationships implying that ammonia oxidation is a conserved trait not subject to HGT and sequence diversity could indicate the origin of the gene thus providing both structural and functional information. Real-time PCR of the *amoA* gene was used to determine the effect of ammonium on population size of ammonia-oxidising bacteria (Okano et al. 2004). This assay, with a detection limit of 1.3×10^5 cells/g of dry soil, demonstrated an increase in population size of ammonia-oxidising bacteria both in soil microcosms and under field conditions. *amoA* was detectable 8 months after ammonium fertilisation. The population size of total bacteria determined by real-time PCR with a universal bacterial probe remained unchanged.

Denitrification involves four reactions with NO_2^-, NO, and N_2O as intermediates, and N_2 as the final product. Each step of this process is catalysed by different reductases, and, in contrast to nitrification, which is achieved by a few well-studied genera of bacteria, a large number of bacteria can reduce nitrate to nitrite in the absence of oxygen. The reduction of NO to N_2O and N_2 is attributed to a more restricted range of groups (Paul and Clark 1989). Most denitrifiers are common heterotrophic soil genera, such as *Pseudomonas*, *Bacillus*, and *Alcaligenes*, which possess NO_3^-, NO_2^-, and N_2O reductases, but some lack NO_3^- reductase, and others (principally

Pseudomonas) lack N_2O reductase. Clearly, it will be difficult to derive structural-functional relationships from such a plethora of species and it is likely that the genes are subject to HGT.

The widespread possession of denitrification genes in many distantly related species of bacteria (and archea) has meant that 16S rRNA-based methods cannot be used to study functional diversity of the process. Thus, only genes encoding denitrification enzymes can provide functional markers for ecological studies. Complete denitrification requires the sequential action of four enzymes; often biochemically distinct enzymes catalyse the same reaction and it is common for bacteria to have only a part of the pathway. This complexity makes it difficult to use a single gene marker to denote denitrification ability. In a recent study, Gregory et al. (2003) used two degenerate primer pairs for the membrane-associated nitrate reductase (*nar*G) together with a 16S rRNA approach for characterisation of a nitrate-respiring bacterial community. Both 16S rRNA and *nar*G phylogenies were only partly congruent suggesting HGT of the *nar*G gene. Results of 16S rRNA-based studies revealed unexpected species richness within a community. The *nar*G sequences amplified directly from TC-DNA gave a very different picture to the culture-based results, some grouping with Gram-negative groups of denitrifiers and others creating a new deep branching clade not affiliated with any known *narG* sequences. The host background for these genes is most likely to be resolved by metagenomics studies.

11.2.5 Biodegradation of Soil Polymers and C1 Compounds

The most ubiquitous polymers of natural origin found in soil are cellulose and chitin. Cellulolytic activity is critical for carbon recycling and chitin plays a unique role as the most abundant source of nitrogen in soil. Chitinolytic ability is ubiquitous among soil microorganisms (and also numerous higher organisms) and chitin is an important source of N in most environments. Many bacteria possess multiple chitinase genes and fungal chitinases may have dual roles in nutrition and hyphal cell wall growth. In order to enrich for such a diverse and complex functional group of soil microbes, baiting has been used to facilitate retrieval of functional genes. Insoluble substrates such as chitin are amenable to this approach and litter-bags have been used to bury and subsequently retrieve substrate in field conditions. Analysis of the substrate by extraction of TC-DNA and RNA provides functional and structural data on major colonists. Degenerate primers were designed to study *chi* gene diversity (Williamson et al. 2000). Primers specific for family 18 subgroup A bacterial chitinases were

used to compare diversity of chitinolytic populations in grassland receiving different treatments (Metcalfe et al. 2002). Clone libraries recovered from TC-DNA extracted from chitin baits buried at the grassland site were dominated by *Arthrobacter*-like *chi* sequences in plots receiving sludge (S) and sludge plus lime (L) treatment, while libraries from control (C) and L plots contained streptomycete-like and *Stenotrophomonas malthophilia*-like chitinase sequences. No *Arthrobacter* isolates were recovered from the baits but they were represented in 16S rRNA libraries from TC-DNA. Diversity of clones recovered was highest in C and L libraries (detecting seven and nine RFLP types, respectively) but low in S and SL libraries (three and four RFLP types, respectively). The highest chitinolytic activity was detected in S and SL libraries suggesting that sludge treatment did not impair degradative chitinolytic activity of soil but had a negative effect on diversity of the soil chitinolytic population. It will not be possible to definitively confirm the identity of the dominant functional groups until the recovery of linked phylogenetic markers with the *chi* sequences is achieved; metagenomics holds promise to realise this objective.

DNA labelled with stable isotopes can be analysed for functional and taxonomic marker genes. Radajewski et al. (2000) pioneered this approach for the study of methylotrophs in soil. *Methylobacterium extorquens* growing on $^{13}CH_3OH$ produced DNA with an increased buoyant density compared with the control growing on unlabelled methanol. Labelled substrate was fed as bait to microbial communities in forest soil microcosms and, after gradient centrifugation and PCR with universal 16S rRNA primers and primers for *mxaF* (encodes the active-site subunit of methanol dehydrogenase), sequence libraries were made from the labelled DNA. Two groups were detected in the labelled DNA fraction, one belonging to the *α-Proteobacteria* and the other resembling members of the *Acidobacterium* division, the former was expected but none of the cultivated members of the latter have been reported to utilise methanol. The DNA/RNA-stable isotope probing (SIP) approach can derive activity data from 16S rDNA analysis because to become labelled the bacteria must be actively utilising the substrate. The analysis of functional genes including *mxaF*, *mmoX* and *pmoA* (active-site subunits of the soluble and particulate methane monoxygenase) can provide more data on the community structure and diversity in addition to confirming community data derived from 16S rRNA analysis, as illustrated by Morris et al. (2002). SIP is a very powerful technique but is not without limitations for field studies mainly due to the following constraints. First, the price of labelled substrates is high and, to achieve reasonable labelling enabling separation of the heavy/labelled and light/unlabelled DNA/RNA using the gradient centrifugation, long incubation with high consumption of labelled substrate is needed. This prolonged incubation represents another limitation of the SIP approach as crossfeeding between

different groups of soil microorganisms allowed detection of labelled carbon from plant metabolized CO_2 in mycorrhizal fungi. Significant enrichment can occur under these conditions. Our opinion is confirmed by the fact that the majority of published SIP experiments were done either using microcosm/reactor studies or groups of microorganisms with special nutrient requirements/abilities (methylotrophs, phenol-degrading bacteria, etc.) or often a combination of both (Radajewski et al. 2000; Manefield et al. 2002; Lueders et al. 2004a,b,c; Ginige et al. 2004). Another approach is to use other bioindicator molecules such as phospholipid fatty acids (PLFAs). The high sensitivity of gas chromatography/mass spectrometry (GC/MS) analysis of PLFAs extracted from soil microorganisms, significant enrichment of ^{13}C content of 16:1ω5 PLFAs was detected indicating selective enrichment of arbuscular mycorrhizal fungi (Johnson et al. 2005).

11.2.6 Bioremediation Activity

Molecular detection of microbial pollutant-biodegrading genes in environmental samples is useful in assessing the potential bioremediation activity of a particular soil. Beller et al. (2002) developed a real-time PCR method to quantify hydrocarbon-degrading bacteria in sediment samples. A gene coding for the alpha-subunit of benzylsuccinate synthase, *bssA*, a catabolic gene involved in toluene and xylene degradation, was used as a target. The real-time PCR method was sensitive over seven orders of magnitude, and was used to study the changes in a toluene-degrading bacterial community. Microcosms were incubated anaerobically with BTEX (benzene, toluene, ethylbenzene, and xylenes) and NO_3^- in the presence and absence of ethanol. Microcosms with the most rapid toluene degradation also had the highest numbers of *bssA* copies. In microcosms with rapid toluene degradation, numbers of *bssA* copies increased 100- to 1000-fold over the first 4 days of incubation.

Catechol 2,3-dioxygenase genes have also been used as indicators for biodegradation ability of compounds such as benzene, toluene, xylenes, phenol, naphthalene, and biphenyl (Mesarch et al. 2000). This technique can be used to accurately and reproducibly quantify catechol 2,3-dioxygenase genes in complex environments such as petroleum-contaminated soil.

The distribution of genes responsible for the degradation of sulphur-containing hydrocarbons was studied in soil samples contaminated with sulphurous oil (Duarte et al. 2001). Genes that encode enzymes involved in the desulphurisation of hydrocarbons, i.e. *dszA*, *dszB*, and *dszC*, were present in all contaminated soil samples, whereas a range of unpolluted soils gave negative results.

Novel hydrocarbon-degrading genes belonging to the class II aromatic ring-hydroxylating dioxygenase (RHD) gene family were detected in soils using PCR (Taylor et al. 2002). This suggested that distinct groups of novel aromatic hydrocarbon-degrading bacteria exist in soils.

Phenol-degrading genes have been detected in TC-DNA (Watanabe et al. 1998). A gene encoding the largest subunit of multi-component phenol hydroxylase (LmPH) was amplified from phenol-digesting activated sludge. Two major *LmPH* gene bands became apparent after the activated sludge became acclimated to phenol.

Herbicides such as atrazine are common pollutants; the degradation potential of two wetland sites was assessed by PCR and Southern hybridisation to detect a gene coding for atrazine chlorohydrolase (Anderson et al. 2002). Sediments from constructed wetlands receiving agricultural run-off tested positive for *atzA* correlating with observed atrazine mineralisation. Sediment from a natural fen that did not show a high level of mineralisation was negative for *atzA* using PCR and Southern hybridisation. This study indicates that the detection of specific degradation genes in TC-DNA is a good predictor of degradation activity

In addition to bioremediation of organic compounds, microorganisms have also been implicated in the bioremediation of heavy metals such as selenium, uranium, cadmium and mercury. Metal remediation can be achieved by precipitation and immobilisation of contaminants (microbes can reduce metal which can result in detoxification and precipitation; e.g. mercury is taken up into the cell and delivered to the NADPH-dependent flavoenzyme mercuric reductase, which catalyses the reduction of Hg^{2+} to volatile, low-toxicity HgO; Nascimento and Chartone-Souza 2003), by biosorption (passive sequestration by interaction with live or dead biological material; bacteria can be genetically engineered to incorporate metal-binding polysaccharides in their cell surface, or to enhance metal transporters with metal binding metallothioneins in the cytoplasm), or by biomineralisation (formation of insoluble metal precipitates by interactions with microbial metabolites thus achieving concentration and reduction in volume of contaminants). A review of this topic was published by Barkay and Schaefer (2001). It is necessary to keep in mind that not all microbial transformation must necessarily result in reduction of toxicity. For example, environmental production of methylmercury is highly undesirable as this represents the most toxic and readily accumulated form of mercury (National Research Council 2000). Success of any microbial transformation also depends on various factors including catabolic capabilities and physiology of the organism, on environmental conditions, nature of contamination, and on societal and economical considerations. The majority of studies on genes involved in remediation have been on bacterial isolates, with very few attempts at in situ detection in complex environments such

as soil. One study on mercury resistance in freshwater illustrated a 29-fold increase in resistance genes in water enriched with mercury compared to control samples (Barkay et al. 1989).

11.2.7 Molecular Detection of Functional Gene Signatures for Detecting Pathogens in Soil

Detection of pathogens and assessment of their activity in the environment is important for ecological studies as well as for public health. A variety of microbial pathogens that infect humans and animals are known to survive in soil, e.g. enteric pathogens such as *Salmonella* sp., *Vibrio cholerae*, *Shigella* sp., C*ampylobacter jejuni*, *Yersinia* sp., *Escherichia coli* O157:h7 (Santamaria and Toranzos 2003) and other pathogens such as *Mycobacterium bovis* (Young et al. 2005). Pathogens enter the soil via excreta of infected wild and domesticated animals, and animal and human waste applied to agricultural land as fertiliser. Landfill leachates present an additional route for human and animal pathogens to enter the soil; in 1993, the US generated 156 million tons of waste, which was sequestered in landfills. Household waste can contain pathogens present in human and animal faeces, food waste and sewage sludge (Santamaria and Toranzos 2003).

Gene Detection

A number of studies have used PCR primers designed to amplify functional genes specific to *Salmonella* spp. Ziemer and Steadham (2003) assessed nine sets of PCR primers targeting *Salmonella* for their specificity with pure cultures of intestinal-associated bacteria prior to their application to *Salmonella* detection in faecal samples. Functional gene targets of PCR primers included a *Salmonella* pathogenicity island I virulence gene, *Salmonella* enterotoxin gene (*stn*), *invA* gene, *fur*-regulated gene, histidine transport operon, junction between *sipB* and *sipC* virulence genes, *Salmonella*-specific repetitive DNA fragment, and multiplex targeting *invA* gene and *spvC* gene of the virulence plasmid. Way et al. (1993) used three sets of oligonucleotide primers to detect *Salmonella* spp. including from inoculated soil samples. *PhoP* primers specific to the *phoP/phoQ* loci of coliform pathogenic bacteria such as *Salmonella*, *Shigella*, *Escherichia coli*, and *Citrobacter* spp. served as presumptive indicators of enteric bacteria. In addition to the *phoP* primers, the *Hin* and the *H-1i* primers, which targeted a 236-bp region of *hin/H2* and a 173-bp region of the *H-1i* flagellin gene, respectively, were used. Both *Hin* and *H-1i* primers are specific to motile *Salmonella* spp. and are not present in *Shigella*, *E. coli*, or *Citrobacter* spp. Further studies on molecular detection of *S. typhimurium* in soil (Marsh

et al.1998) using Way's primers illustrated *H-Li* PCR products at $\geq 10^3$ cells g^{-1}. It was also demonstrated using QPCR that *S. typhimurium* numbers decreased over time in non-sterile microcosms, from 10^8 copies of the *H-Li* gene g^{-1} soil at day 0 to 10^5 copies g^{-1} soil at day 60.

Faecal coliforms were detected in inoculated soil samples by amplification of a 179-bp fragment of *lamb*, a gene that encodes an outer membrane protein present specifically in faecal coliforms. Following cell extraction with $CaCl_2$ and purification using sucrose density gradient centrifugation, 1 cfu of *E. coli* was detectable in 1 g of soil (Josephson et al. 1991).

Different soils require different methods to efficiently extract DNA. Poussier et al. (2003) applied a wide range of published methods, modifications and novel methods in order to detect *Ralstonia solanacearum*, the causative agent of bacterial wilt in solanaceous plants, from four different soil types. Protocols using the QIAamp kit (Qiagen, Courtaboeuf, France) were found to be most reliable.

E. coli O157:H7 is an important human pathogen found in soil, and the possibility of prolonged survival has led to the development of novel methods used to detect virulence genes which differentiate it from non-enterohaemorrhagic strains. *E. coli* O157:H7 has been detected in inoculated soil and wetland samples using real-time PCR (Ibekwe et al. 2002). Shiga-like toxin genes *stx1*, *stx2* and the intimin gene *eae* were detected in inoculated soils. DNA was extracted using an UltraClean soil DNA kit (MO BIO). Sensitivity of the multiplex PCR was established as 7.9×10^{-5} pg of starting DNA ml^{-1} equating to 6.4×10^3 cfu ml^{-1} in genomic DNA; in DNA extracted from seeded soil samples, the detection limit ranged from 3.5×10^3 cfu ml^{-1} for the *stx1* gene and 3.5×10^4 cfu ml^{-1} for the *stx2* and *eae* genes.

PCR primers specific for the *Mycobacterium tuberculosis* complex have been used to detect the presence and survival of *M. bovis* BCG (Pasteur) in soil microcosms and subsequently *Mycobacterium bovis* in the environment (Young et al. 2005). PCR detection of the *M. tuberculosis* complex in clinical specimens has been achieved using antigen genes, such as *mpb70* (Gormley et al. 1999) and also the insertion sequence IS*6110* (Broccolo et al. 2003). The former provides a highly specific and quantifiable target for molecular detection, as it is a single copy gene found only in members of the tuberculosis complex. Environmental samples were collected from a farm in Ireland with a history of bovine tuberculosis and *M. bovis* was detected in soil up to 21 months after initial contamination. *M. bovis*-specific 16S rRNA sequences were detected, providing evidence of the presence of viable cells in the Irish soils. Studies of DNA turnover in soil microcosms proved that dead cells of *M. bovis* BCG did not persist beyond 10 days. Further microcosm experiments revealed that *M. bovis* BCG survival was optimal at 37 °C with moist soil (−20 kPa, 30% v/w).

The use of microarrays for the detection of pathogens has become more common in recent years, although few have applied the technology to detection in complex environments such as soil. Chizhikov et al. (2001) used microarray analysis to discriminate strains of *E. coli* and other pathogenic enteric bacteria harbouring various virulence factors. The presence of six genes (*eaeA*, *slt-I*, *slt-II*, *fliC*, *rfbE*, and *ipaH*) encoding bacterial antigenic determinants and virulence factors was monitored by multiplex PCR followed by hybridisation of the denatured PCR product to the gene-specific oligonucleotides on a microchip. The array was able to detect virulence factors in 15 *Salmonella*, *Shigella*, and *E. coli* strains. The advantage of this technique over conventional visualisation using gel electrophoresis was that the latter technique produced unexpected and uncharacterised PCR products giving ambiguous results. Lievens et al. (2003) used a DNA array for the rapid detection of multiple tomato wilt pathogens using internal transcribed spacer (ITS) sequences from rDNA. Seeded potting compost was inoculated with fungal cultures and then tomato seedlings were planted and grown for 7 and 10 weeks. The pathogens were subsequently detected in compost using the DNA array.

Metabolic Activity and Infectivity of Bacterial Pathogens in Soil

The physiological state of pathogens in soil is largely unknown. There is evidence that organisms such as salmonellae become "viable but non-culturable" (VBNC) in soil (Turpin et al. 1993) with a reduction in cell size. However, laboratory microcosm studies have shown that different serovars of *Salmonella* and *E. coli* are able to grow in composts at 37 °C. Agricultural soils are highly heterogeneous, with areas of variable organic matter (e.g. faeces, decaying plant material) so the possibility remains that an active subpopulation of pathogenic bacteria may exist in the soil environment. Intracellular growth within soil protozoan vacuoles has been demonstrated for some bacterial pathogens, from where bacterial cells can be released and provide an active subpopulation of cells in soil (Barker et al. 1999; Gaze et al. 2003).

The detection of genes from pathogenic organisms in soil gives limited information regarding the viability, metabolic activity or infectivity of the organisms. DNA remains amplifiable by PCR for some time after cell death. Romanowski et al. (1993) reported that, after 60 days, 0.2, 0.05, and 0.01% of added genes on plasmids were detectable by PCR in a loamy sand soil, a clay soil, and a silty clay soil, respectively. However, in seeding experiments with *M. bovis* DNA, lysed cells and dead cells, no DNA could be detected after 10 days indicating that detection of DNA was a useful indicator for the presence of live cells in soil from fields known to have been grazed by infected cattle (Young et al. 2005). The half-life of mRNA molecules is

considerably shorter than that of DNA, and many studies have concentrated on detection of these molecules as evidence of pathogen activity in soil. Laboratory studies have shown that mRNA in *E. coli* becomes undetectable after 2–16 h after being heat-killed (Sheridan et al. 1998); mRNA has been extracted directly from soil and has proved useful in investigating microbial activity (Sessitsch et al. 2002; Bürgmann et al. 2003; Anukool et al. 2004). However, very little work has been undertaken applying this technique to pathogens in soil.

In situ RT-PCR has allowed the physiological state of individual *S. typhimurium* cells to be monitored. Holmstrom et al. (1999) used *groEL* mRNA (which is induced by heat shock) and *tsf* mRNA (which is expressed as a function of growth rate) to monitor different physiological functions. Intracellular mRNA was amplified using biotin-labelled primers in fixed cells and PCR products were detected using a streptavidin-horseradish peroxidase conjugate; this type of approach has considerable potential for elucidation of pathogen activity in soils. Nucleic acid based fluorescent probes can also be used to monitor bacterial cells extracted from soil in conjunction with flow cytometry; several reviews are available on the subject (Davey and Kell 1996; Amann and Kuhl 1998; Porter and Pickup 2000; Shapiro 2000; Steen 2000).

The expression of virulence genes may not occur when a pathogen is outside its host, and the retention of virulence can only be confirmed by use of infectivity assays using animal models. The virulence of cells extracted from soil and identified using oligonucleotide probes and FACS could theoretically be tested. Some research to date suggests that pathogens such as *Vibrio* spp. which appear to be in a VBNC form are capable of resuscitation and retain their infectivity (Baffone et al. 2003).

11.3 Expression of Functional Genes in Soil

11.3.1 Introduction – Methods for the Detection of mRNA in Soil

Expression of functional genes in soil can be monitored by detection of their transcription either directly as mRNA or via a reporter gene fused to the target gene. For interpretation it is important to know the mechanism of regulation for expression of the activity of interest, as levels of mRNA may not always be indicative of enzyme activity due to posttranscriptional modification.

To quantify, or detect in situ, a specific cell population or gene activity in soil, a marker gene (expressed from a constitutive promoter resulting in

a detectable phenotype) or a reporter gene (expressed from an inducible promoter) can be used (see Chaps. 16–18)

Luminescent marker genes *luc* and *lux*AB, derived from eukaryotic and bacterial luciferase, respectively, have been used in microbiological studies; due to limitations such as energetic demands on cell metabolism, their usefulness for studies of soil microbial populations is limited. Consequently, fluorescent marker genes, mainly those encoding fluorescent proteins such as the green fluorescent protein (GFP), have been used, despite limitations such as a requirement for molecular oxygen and a need for specialised detection equipment. In contrast to luminescent markers, fluorescent markers place less demands on cell energy. Chromogenic reporter genes have also been used in many environmental studies, including: *xyl*E encoding catechol 2,3-dioxygenase, *lac*Z for *β*-galactosidase, *gus*A encoding *E. coli* glucuronidase, *ina*Z encoding ice nucleation activity, and different antibiotic resistance genes such as *npt*II which produces kanamycin resistance. Most bioreporters use an environmentally or metabolically responsive promoter, which is fused to a suitable reporter gene and introduced into a microbial host. Host cells then react to environmental stimuli by production of an easily detectable reporter protein (Leveau and Lindow 2002; Hinde at al. 2003; Jansson 2003).

Another fluorescent method for the in situ detection of a gene of interest is based on hybridisation of a target gene with a fluorescently labelled probe (Bakermans and Madsen 2002; Ginige et al. 2004). Fluorescent in situ hybridisation (FISH), where 16S rRNA sequences are targeted with specific fluorescently labelled probes to identify active bacteria (having a higher ribosomal count) within a community population, has been widely used in different environments. Studies in the soil environment have been limited due to autofluorescence of soil particles and the small size of indigenous bacteria which show only dim fluorescence (Hahn et al. 1992; Christensen et al. 1999). Metcalfe (2002) used this technique to specifically detect cells of chitinolytic *Stenotrophomonas maltophilia* in a cell extract from chitin bags buried in soil for a period of a few weeks. Comparing cell extracts from July and November sampling, the July extract showed brighter cells indicative of higher activity. This finding corresponded with higher chitinolytic activity detected in the July sampling.

FISH was recently coupled with autoradiography (MAR-FISH/STAR-FISH/MICRO-FISH) enabling simultaneous microscopic determination of in situ identities, activities, and specific substrate uptake of bacterial cells within complex bacterial communities (Lee et al. 1999; Ouverney and Fuhrman 1999; Cottrell and Kirchman 2000; Gray et al. 2000; Ito et al. 2002). These methods are based on feeding of a natural community with a radioactive substrate. After incubation cells are fixed on a slide and subjected to FISH treatment and microautoradiography. Using fluorescently

labelled oligonucleotide probes with different levels of specificity, identity of individual cells can be determined. As the same slide is also used for the autoradiography, affiliation data from FISH can be connected with activity data obtained by autoradiography.

The original FISH method relies on the presence of many target sequences (16S rRNA) within an individual cell. This was later modified to detect a single copy of a functional gene in a cell. Tani et al. (1996) used this method to identify cells of *E. coli*. Cells were spotted on a slide, the cell wall permeabilised by lysozyme and proteinase K, and PCR performed directly on the slide using a digoxigenin-labelled primer. The digoxigenin-labelled product was then detected with a labelled anti-digoxigenin antibody and visualised using a suitable technique such as epifluorescent microscopy or confocal laser-scanning microscopy (Tani et al. 1996; Hoshino et al. 2001). The same principle was used for the detection of mRNA in a bacterial cell by Hodson et al. (1996). After permeabilisation of the cell wall, RT-PCR was carried out; cells were washed and used in a second PCR with the labelled primer.

A variety of methods have been used to detect expression of functional genes in the pool of community RNA extracted from a particular environment (ex situ). The first step is the isolation of mRNA from soil. Traditionally, it was believed that mRNA was short-lived and nearly impossible to isolate. This was based on studies of the turnover of prokaryotic mRNA, where, for example, the half-life of specific *E. coli* mRNAs ranged from about 0.5 to 20 min, which was constant with doubling times between 60 min and 10 h (Nierlich and Murakawa 1996). However, it has been shown that slower growing environmental strains have longer mRNA half-lives (Fleming and Sayler 1995), and the situation is rather different with eukaryotic mRNA. In prokaryotes, mRNA is translated nearly simultaneously as it is transcribed and often many genes are transcribed together in a single polycistronic mRNA, whereas transcription and translation in eukaryotes is physically separated by the nucleus membrane resulting in temporal separation. Eukaryotic mRNA also requires splicing of intervening sequences before it can be translated, which means that it has a longer half-life measured in hours rather than minutes (Sarkar 1997; Rauhut and Klug 1999). Further problems hampering RNA isolation are associated with the isolation process itself. After cell lysis RNA can be quickly degraded by the action of ubiquitous RNases, or bound to soil particles and colloids via functional groups of the nitrogenous bases of single-stranded RNA (Ogram et al. 1994, 1995; Mendum et al. 1998). Despite these technical challenges several methods for the isolation of RNA from natural environments have been published (Selenska and Klingmüller 1992; Ogram et al. 1995; Griffiths et al. 2000; Hurt et al. 2001; Sessitsch et al. 2002; Bürgmann et al. 2003). Once isolated, various methods for RNA analysis are available. One of the traditional methods

which has proved to be fast and reliable is a slot or dot blot hybridisation, where RNA is applied to a membrane and subsequently probed with a ^{32}P-labelled in vitro transcribed RNA probe. A comprehensive review of such methods can be found in Sayler et al. (1992).

To detect differences in gene expression in a bacterial strain under different conditions or between different strains, subtractive hybridisation can be used. cDNA prepared from total RNA of a wild type or a strain cultivated under certain conditions is hybridised with RNA from a mutant or strain cultivated under different conditions. Double-stranded cDNA-mRNA hybrids are then selectively removed and the composition of single-stranded cDNA left in the supernatant is analysed (Utt et al. 1995).

Hybridisation is also used in ribonuclease protection assays, where an extracted soil RNA is hybridised to a labelled, in vitro transcribed anti-sense RNA probe complementary to the gene of interest. The non-hybridised single-stranded probe is then removed by ribonuclease action and the protected double-stranded RNA hybrid is quantified by comparison with standards (Fleming et al. 1993).

A differential display method widely used for eukaryotic gene discovery takes advantage of the polyadenylate tail present on most eukaryotic mRNAs. Despite the fact that poly(A) polymerase was isolated from *E. coli* as early as 1962, and again during the 1970s from other bacteria, most recently in *Streptomycetes* (Sarkar 1997; Rauhut and Klug 1999; Jones 2002), it was long believed that polyadenylation was a characteristic of eukaryotic mRNA and quite rare in bacteria. Now it is believed that 2–50% of bacterial mRNA have poly(A) tracts of 14–60 nucleotides, in comparison with 100% polyadenylation (80–200 nucleotides long) in eukaryotes. The exact role of polyadenylation is still not fully established but there are indications that the poly(A) tail plays a role in the stability of mRNA.

The 3′ primer used in the first step of the differential display method, reverse transcription, and also for the following PCR consists of a poly(T) region with two additional bases that recognise one-twelfth of the total mRNA population. The 5′ primer is arbitrary and short (6 to 7 bp) and should ideally anneal within 500 bp of the 3′ primer; this means that, in contrast to other methods, there is no need for a priori information on gene sequences for design of specific probes or primers. Resulting PCR products are resolved by a DNA sequencing gel creating a ‘fingerprint’ of the studied population. A low annealing temperature (42 °C) is used and specificity of DNA amplification can be increased by decreasing the concentration of deoxynucleoside triphosphates (Liang and Pardee 1992). The method can be used to visualise mRNA compositions of cells, cDNAs can be sequenced, or individual bands can be cloned and used as probes for Northern or Southern blotting. Reproducibility is very high (95% of bands were always seen). To study bacterial R/DNA lacking the poly(A)

tail, both primers can be arbitrary. Wong and McClelland (1994) used this method searching for stress-induced RNAs in *Salmonella typhimurium*, and Fleming et al. (1998) detected differential mRNA transcription both from pure-culture and soil-derived bacterial DNA. After optimisation of the primer concentration, length, annealing temperature, concentration of template, deoxynucleotide triphosphate, and $MgCl_2$ concentration, the detection limit of *tod*C1 was 0.015 ng of total RNA template or approximately 10^3 transcripts. Another possible variation of this method is the use of "motif" primers using a sequence common for a group of genes of interest where the PCR product is then biased towards this group (Stone and Wharton 1994). An important limitation of the method is that less abundant RNAs will probably be under-represented among the visible products on the gel. A possible solution to this problem might be a nested PCR with identical primers with one or more extra arbitrary bases at the 3′ end. The most vexing problem of this method is the occurrence of false positives. Less than half of the putative differentially expressed PCR products excised from gels were truly differentially expressed.

Competitive PCR can be used to determine the relative transcript level of a gene of interest. After the isolation of RNA, RT-PCR using a gene-specific primer is run. For the second PCR a series of dilutions of competitive templates (genomic DNA) is added and resulting products are separated on an agarose gel according to their size, as PCR products of the competitive template are longer due to the presence of introns (Lamar et al. 1995; Bogan et al. 1996a). Recently, Han and Semrau (2004) coupled RT-PCR with capillary electrophoresis, which resulted in higher sensitivity of the detection of RT-PCR products. The method is also very fast, requires only small amounts of sample, and can be completely automated from sample injection to data analysis.

Real-time PCR uses a double-labelled probe to measure the accumulation of fluorescence of a released reporter dye during PCR, which is proportional to the amount of product formed during PCR and can be correlated to the original amount of template DNA or even mRNA. This method is highly suitable for quantification of transcripts of genes of interest (Rodrigues et al. 2002).

Detection of Key Metabolic Processes in Soil

Metabolic processes of soil microorganisms are of key significance due to their ecological and economic importance; one important metabolic process is antibiotic production/resistance. A large proportion of antibiotics currently in use are of actinomycete or fungal origin, therefore detection of genes involved in their production and understanding expression is of a great importance. In addition to detection of antibiotic synthesis genes,

detection of antibiotic resistance genes is becoming more important in light of the increasing resistance seen in environmental and clinical bacteria.

Another important group of microbial genes are those involved in the N cycle. Nitrogen is the main element limiting plant productivity in nearly all natural systems. The efficient use of N available to plants is essential regardless of whether it is N fixed by symbiotic or free living N-fixing bacteria, or N in organic residues entering soil which has to be released by microbial activity (mineralisation), or N delivered in industrial fertilisers, where the efficiency of its use is important due to the cost of fertiliser production. In addition to the economic importance of efficient utilisation of N fertilisers, ecological impact must be taken into account as high levels of N released in the form of NO_3^- can contaminate ground water and plant crops.

Degradation processes are also of interest, as all organic matter produced must be decomposed to release bound elements back to the element cycles. The soil microbial population conducts a major part of these processes. Diversity of degradative abilities of soil microbes even ensures degradation/bioremediation of anthropogenic substances entering the natural environment.

Expression of Antibiotic Activity, Antagonism

A *lux*AB reporter gene was used to monitor the expression of the antibiotic phenazine-1-carboxylic acid (PCA) by Seveno et al. (2001). The *lux*AB from *Vibrio harveyi* was inserted in the *phzB* gene of the phenazine operon and transcription was monitored by measurement of luminescence in liquid culture, on nutrient agar, on sterile wheat seedlings and in sterile bean rhizosphere. Production of phenazine was confirmed both in liquid culture and on solid media, but it could not be detected on wheat seedlings or in bean plant root rhizosphere despite the fact that transcription of the *phz*B::*lux*AB reporter occurred on the bean plant root.

Direct detection of mRNA of an antibiotic production gene using RT-PCR was achieved by Anukool et al. (2004). Streptothricin (ST) production by *Streptomyces rochei* F20 was monitored in liquid culture, soil and the rhizosphere of spring wheat. After RNA extraction, primers specific for the ST resistance gene (*stt*R) and ST biosynthesis gene (*stt*A) coding for peptide synthetase of *S. rochei* were used for RT-PCR. A higher level of expression of *sst*R in comparison with *stt*A was detected in all experiments. mRNAs of both genes were detected in soil containing approximately 10^6 cfu g^{-1} soil. The *stt*R mRNA was detected both in sterile and non-sterile rhizospheres, but neither *stt*R nor *stt*A transcripts were detected in the rhizoplane.

The role of antibiotics in biological control has been reported (Rothrock and Gottleib 1984; Brisbane and Rovira 1988; Thomashow et al. 1990; Howie and Suslow 1991). Detection of antibiotics in soil is difficult due to low level

production and adsorption to clays. Thiostrepton production in sterile soil was determined by Wellington et al. (1993) within the range of 30–50 ng g^{-1} soil following extraction and bioassay via a specific thiostrepton-inducible promoter coupled to a resistance gene. A highly sensitive inducible promoter driving *gfp* expression was used to detect oxytetracycline production in soil where FACS analysis enabled direct counting of fluorescing cells in soil extracts (Hansen et al. 2001). The direct detection of phenazine in the wheat rhizosphere after inoculation and incubation with *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 proved the significance of antibiotic production in control of Take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Brisbane and Rovira 1988).

Detection of Expression of N Cycle Genes in the Environment

Gene expression can be correlated to bulk N fixation. Bürgmann et al. (2003) extracted *nif*H mRNA from *Azotobacter vinelandii* growing in liquid culture and sterile soil, and using RT-PCR estimated expression. Bhagwat and Keister (1992) used the subtractive RNA hybridisation procedure to characterise genes responsible for increased competitiveness for nodulation of the strain *Bradyrhizobium japonicum* USDA 438 in comparison with the strain USDA 110. Both genes were isolated and characterised.

Expression of five denitrification genes in two estuarine sediments was studied by Nogales et al. (2002). The presence of all five genes was confirmed by PCR in both sediments although RT-PCR detected transcript of only two genes, the *nir*S and *nos*Z genes, the latter only by Southern hybridisation. Consistently stronger RT-PCR products from one sampling site corresponded with a 10-fold higher plate count of denitrifiers from this site indicating the semi-quantitative nature of RT-PCR. The failure to detect all five denitrifying genes on the RNA level could be due to slightly different specificity of primers, inhibitory effects of co-purified impurities (mainly humic substances) in RNA samples, preferential amplification of certain templates, or insufficient quantity of template needed for detection. Cloning and sequencing of *nir*S RT-PCR products revealed a high diversity of the gene in both environments, where 13 of 16 clusters appeared novel suggesting the presence of unknown denitrifying bacteria. Most sequences obtained were specific to single sampling sites emphasising the high diversity of denitrifiers.

The community structure of ammonia-oxidising bacteria within anoxic marine sediments was studied by Freitag and Prosser (2003). The communities were studied by denaturing gradient gel electrophoresis (DGGE) analysis and sequencing of 16S rRNA genes using group-specific primers by PCR and RT-PCR. The RT-PCR was used in parallel with PCR to achieve greater sensitivity due to the increased number of targets provided by the

ribosomes and also indicated which members of the community were most metabolically active. RNA-derived DGGE banding patterns were similar to those derived from DNA, but not all dominant bands present in rDNA were detected, suggesting differences in growth and activity of ammonia-oxidising bacteria. This may reflect the heterogeneity in physicochemical characteristics within the sediments.

Expression of Decomposer Activity

The activity of microbial decomposers can be elucidated using various techniques. Transcription of the cellulase/hemicellulase genes of *Clostridium cellulovorans* were studied using Northern blot, RT-PCR, primer extension and nuclease protective assay (Han et al. 2003). Northern hybridisation illustrated that the cellulosomal *cbpA* cluster was transcribed as a polycistronic mRNA of 8 and 12 kb. Primer extension and nuclease protective assay located transcriptional start sites of four genes in the cluster. Expression of ligninolytic enzymes (*lip*) of *Phanerochaete chrysosporium* in wood was studied by Janse et al. (1998). Poly(A) RNA was isolated by magnetic capture and the specific transcriptions of all ten *lip* genes plus three manganese peroxidase genes (*mnp*) were quantified by competitive RT-PCR with full-length genomic subclones. Differences in the transcript level ranged up to 10,000-fold and the transcript pattern of different lignin peroxidase genes expressed in wood differed dramatically from those previously obtained with defined media or from soil cultures, indicating specialisation of different enzymes to certain conditions. In addition, Stewart and Cullen (1999) studied transcription of *lip* genes of *Phanerochaete chrysosporium.* Using competitive RT-PCR they assessed *lip* transcript levels in both C- and N-limited media, which showed differential regulation of *lip* genes and the presence of constitutive and up-regulated transcripts. Iron-responsive genes of *P. chrysosporium* were studied by Assmann et al. (2003). Using differential display RT-PCR they identified 97 differentially expressed cDNA fragments, the majority of them encoding proteins involved in iron acquisition.

Bioremediation Activity

Microbial remediation of pollutants has been measured by quantifying messages from inducible genes involved in degradation. Selvaratnam et al. (1993) used RT-PCR to monitor the 3-chlorobenzoate-degrading catabolic *tfd*B gene of *Pseudomonas putida* inoculated into non-sterile activated sludge, and the phenol-degrading *dmp*N gene of *P. putida* in a sequencing batch reactor. Expression of *dmp*N correlated with the degradation of phenol, confirming the suitability of this method for monitoring and controlling operation of a reactor.

Degradation of polycyclic aromatic hydrocarbons (PAHs) in sterile soil microcosms was investigated using competitive RT-PCR for the detection of mRNA of three manganese peroxidase genes (*mnp*) of *Phanerochaete chrysosporium* (Bogan et al. 1996a). Levels of *mnp* mRNA corresponded with the disappearance of high ionisation potential PAHs during soil microcosm experiments. This confirmed that monitoring levels of mRNA could be used as an indicator of physiological state of the fungus under real conditions during remediation processes.

Mechanisms of naphthalene degradation have been studied by quantification of m-RNA transcripts of *nah*A. A naphthalene-lux reporter system was also used to monitor degradation of naphthalene and determine bioavailability within soils heavily contaminated with PAHs. The naphthalene mineralisation rate correlated positively with the number of *nah*A gene transcripts present and the naphthalene-lux reporter system confirmed bioavailability of naphthalene illustrating the usefulness of this monitoring system (Sanseverino et al. 1993–94). Bakermans and Madsen (2002) used FISH with tyramide signal amplification (TSA), routinely used to detect mRNA in eukaryotic systems, for the detection of intracellular mRNA for the naphthalene dioxygenase gene (*nahAc*) in naphthalene-degrading *P. putida* NCIB 9816-4. TSA significantly increased probe fluorescence and only 100–1000 copies of the hybridisation target molecules per cell were needed for detection. The efficiency of the method was tested on naphthalene-contaminated groundwater samples. Positive results represent progress towards the main goal of environmental microbiology, to simultaneously detect identity, activity and biogeochemical impact of microorganisms in situ, including in soil, water, and sediments.

To detect mercury in the environment, Hansen and Sørensen (2000) constructed three cell biosensors fusing the mercury-inducible promoter, P_{mer}, and its regulatory gene, *merR*, with reporter genes *luxCDABE*, *lacZYA*, or *gfp*. Transferred into *P. putida*, this reporter system was able to quantitatively detect mercury in contaminated soil. The construct was cloned into a mini-Tn5 delivery vector which was able to transfer into a variety of Gram-negative bacteria. Jeffrey et al. (1996) extracted mRNA from the concentrated bacterial fraction from a freshwater pond and detected the transcript of the *merA* gene by probing with a radioactive antisense probe. The quantity of the transcript did not correspond with the concentration of dissolved mercury but rather with activity of the microbial community.

11.4 Linking Enzyme Activity to Gene Expression

11.4.1 Introduction

Linking enzyme activity to gene expression in soil is a challenging task. Detection of an enzyme activity in soil only indicates the presence of a protein capable of performing a certain biochemical reaction regardless of its origin. The enzyme may be intra- or extracellular, and newly synthesised or be part of the soil enzyme pool absorbed to the soil matrix composed of clay minerals, organic matter and humic substances (see Chaps. 4 and 5). Enzymes can be of microbial, plant or animal origin. Even if it is possible to determine the microbial origin of an enzyme, there are usually many microbes capable of producing the same enzyme. In addition, there are often multiple genes coding for enzymes able to catalyse the same or similar reactions, leading to some functional redundancy, increasing the capacity of the producing organism to adapt and deal with a variety of environmental conditions (Naessens and Vandamme 2003).

The first challenge is the extraction of protein from soil, either as a crude or relatively purified enzyme extract. Several procedures for extraction of enzymes from soil have been described and differ in composition of the extraction buffer, pH and temperature; methods usually involve some kind of mechanical treatment for a variable period of time if intracellular enzymes are to be assayed. Once extracted, different purification processes can be applied. Tabatabai and Fu (1992) and later Nannipieri et al. (1996) summarised many different approaches to the isolation and purification of enzymes from soil, and in this volume the matter is discussed in Chaps. 4 and 5.

11.4.2 Decomposer Activity and Bioremediation

The ligno-cellulose complex accounts for a substantial part of plant biomass; it is the most abundant biopolymer in nature. Degradation of such polymers is of a great importance in the maintenance of nutrient cycles. There have been many studies dealing with the enzymology of these processes or their genetics, but few have attempted to link activity to specific gene products. Bogan et al. (1996a) quantified transcripts of all ten known lignin peroxidase genes (*lipA–lipJ*) of *Phanerochaete chrysosporium* in soil microcosms amended with 400 ppm of anthracene. After extraction of mRNA the presence of *lip* gene transcripts was detected and quantified

by competitive RT-PCR. Lip proteins were extracted from microcosms, purified and applied to a nitrocellulose membrane. Western blotting was performed with monoclonal antibodies to *P. chrysosporium* LiP H8. Levels of extractable *lipA* transcript and protein (LiP H8) were well correlated, although they were separated by a 2-day lag period. During subsequent studies, Bogan et al. (1996b) were able to detect transcripts of nine *lip* genes, even in non-sterile soil from a hazardous waste site. These reports demonstrate the utility of the method for accurate assessment of the physiological state of *P. chrysosporium* during soil bioremediation processes.

An attempt to cover the whole process of gene expression starting from DNA through mRNA, enzyme production and its detection in soil environment was done by Metcalfe et al. (2002). The method of buried litter-bags filled with crab-shell chitin was used to study the composition of a soil chitinolytic community and its changes under different treatments (lime and sewage sludge application). Enzyme activity was determined by measuring weight loss of chitin in bags and using a luminescence assay with a 4-methylambelliferyl-$(GlcNAc)_2$ analogue of chitin. Community structure was assessed by DNA extraction, cloning and sequencing of PCR products using primers for family 18 group A chitinases. Unfortunately, attempts to extract RNA failed probably due to the well-known absorption properties of chitin. The highest chitinolytic activity was detected in sludge-treated plots, which corresponded with greater number of actinobacteria in these plots. Sequence analysis showed major differences in the community structure under the influence of sludge and lime treatment. Despite the failure to conduct mRNA studies, which would have identified the active part of the chitinolytic community present in bags, the study demonstrated that the method of buried litter-bags can yield useful data. The addition of substrate can enrich for a population of interest facilitating study of the degradative potential of a soil microbial community. If the substrate is readily retrievable the active community can be studied by a variety of molecular techniques. It may be possible to link detected enzymes to producing microorganisms using comparisons of DNA/mRNA analyses with sequences obtained from the reverse genetics of proteins. A major obstacle here will be the quantity and purity of active enzyme proteins extractable from soil but there are powerful techniques for protein purification and sensitive analysis (see Chaps. 4 and 5).

Mass spectrometry-based proteomics was used to analyse proteins isolated from dissolved organic matter (DOM) by Schulze et al. (2005). The aim was to identify proteins and determine their origin, in addition to detecting extracellular enzymes possibly important in the carbon cycle. Identified proteins were classified according to their phylogenetic origin. Data indicated that a higher proportion of fungal proteins were isolated from forest soils. Enzymes involved in degradation of organic matter were

not identified in free soil DOM although cellulases and laccases were found among proteins extracted from soil particles, indicating that degradation of soil organic matter takes place in biofilms on particle surfaces.

11.5 Conclusions

The last two decades have provided significant developments in our ability to define microbial populations in soil in terms of their functional potential, as reviewed by Wellington et al. (2003). However, the most significant progress has been in the discovery of the very extensive diversity within soil microbial communities. It is possible to resolve within the tree of life new nodes and clades of previously unknown and uncultured bacteria or fungi, although understanding their function in situ still presents considerable technical challenges. Ecologically significant and useful data is being collected following analysis of functional gene families in soil, but we are still limited to what we know from studies of functional genes in culturable soil isolates. The diversity in such genes will always result in PCR-based detection methods missing the more diverse populations. The lack of conservation and parallel evolution within protein-coding genes, which are not essential for replication and maintenance, will cause even more difficulties in gaining a representative view of functional diversity. It is unlikely that the labour-intensive metagenomic library approach will solve these problems as it is a considerable challenge to provide a representative library of >8000 distinct genomes which may be present in some soils. A major objective of studying genetic diversity in situ within the soil is to understand the soil's capacity for supporting plant and animal communities and assessing the impacts of xenobiotics and agricultural activities. The advent of techniques such as SIP (see Chap. 10) and adding labelled substrates to soil are likely to provide focused but more meaningful studies of the activities of soil bacteria and fungi.

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12 Enzyme Activities in Soil

Liliana Gianfreda, Pacifico Ruggiero

12.1 Introduction

There is an extensive bibliography on the occurrence and distribution of enzymes in different soils, the measurement of their activity, their catalytic performance, the stability and/or the sensitivity of soil enzyme activities to natural and anthropogenic factors, the role of soil enzyme activities in the whole response of a soil to a disturbance, etc. Although three entire books (Burns 1978a; Kiss et al. 1998; Burns and Dick 2002) and several book chapters (Skujins 1967, 1976; Kiss et al. 1975; Burns 1978b, 1986; Ladd 1985; Nannipieri et al. 1990, 2002; Dick and Tabatabai 1992, 1999; Dick 1994; Nannipieri 1994; Tabatabai 1994; Dick et al. 1996a; Gianfreda and Bollag 1996; Ruggiero et al. 1996; Tabatabai and Dick 2002) have dealt with a number of these aspects, several uncertainties are still present and several drawbacks are not solved in soil enzymology, especially with regard to the interpretation of measurements of enzyme activities and the implications of these measurements in soil functioning.

In this chapter some of these shortcomings will be addressed and some possible solutions will be discussed. To avoid repetitions the authors have preferred to examine recent literature. However, indications of fundamentals and achieved aspects of this subject are reported when required.

12.2 Type, Distribution, Location and Properties

Microbial, plant and animal cells can be present in soil and release enzymes upon cell death and lysis or for physiological reasons such as hydrolysis

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of polymers in oligomers or monomers to be taken up by cells. Therefore, soil is rich in enzymatic proteins which catalyse reactions involved in energy transfer, nutrient cycling, environmental quality and crop productivity (Dick 1994; Tabatabai 1994). Thus, the number of enzymes in soil is expected to be very high and at least 500 enzymes can have a critical role in both C and N cycles. However, the number of enzyme activities so far measured in soil is much more limited (Dick et al. 1996a; Gianfreda and Bollag 1996; Nannipieri et al. 2002). For instance, the enzymatic activities involved in the N cycle, and identified in soil, are summarised in Table 12.1. This number is by no means exhaustive, considering all N transformations occurring in soil.

Some of the enzyme activities listed in Table 12.1 were detected many years ago (Skujins 1967, 1976; Burns 1978a), whereas others have been measured only recently (Acosta-Martinez and Tabatabai 2000, 2001). Most of them were tested by using synthetic rather than natural substrates. This along with other methodological aspects may lead to an erroneous evaluation of the meaning of enzyme measurements in soil, as discussed later.

The majority of the examples reported earlier (Gianfreda and Bollag 1996; Nannipieri et al. 2002) and those summarised in Table 12.1 refer to enzyme-like activities of soil rather than to activities of purified enzyme proteins. Unlike what occurs with other living systems, very few enzymes or enzyme-like activities have been extracted from soil and their purification has been generally unsuccessful (Mayaudon 1986; Tabatabai and Fu 1992; Nannipieri et al. 1996; Chap. 4). Generally, these enzymes have been extracted as organo-enzyme complexes, i.e. as enzyme activities associated with organic material (see Chap. 4).

Various intracellular and extracellular enzymatic forms contribute quantitatively and qualitatively to the overall enzymatic activity of soil (Burns 1978b, 1982). The same enzyme can have a different origin (i.e. from bacteria, fungi, plants, and a range of macroinvertebrates) and occupy different locations not only in the producing cell, but also in the extracellular soil matrix (Burns 1978b, 1982). The same enzyme can be intracellular or associated to the external surface of the originating cell (cells can be alive, dead, proliferating or non-proliferating; active enzymes can be also associated to cell fragments), and, if secreted outside the cell, they can be free in soil solution, adsorbed by clays or entrapped by humic molecules. Thus it may have different properties, and show different behaviours, in response to a given factor.

Generally, the activity of free enzymes is considered very low and thus its contribution to the measured enzyme activity can be neglected also considering their short life as free proteins in an inhospitable environment such as soil (Nannipieri and Gianfreda 1998). The fact that free extracellular

Table 12.1. Soil enzyme activities involved in the N cycle

Enzyme (trivial name)	Function	Natural substrates	Substrates used in the assay	References
Amidase (EC 3.5.1.4)	Hydrolysis of C–N bonds other than peptide bonds in linear amides	Peptides, amides	Formamide	Miller et al. (2001); Dodor and Tababatabai (2003)
Arylamidase (EC 3.4.11.2)	Hydrolysis of a N-terminal amino acid from peptides, amides or arylamides	Arylamides	L-Leucine	Acosta-Martinez and Tabatabai (2001, 2002); Dodor and Tabatabai (2002); Tabatabai et al. (2002)
Peptidases	Peptides hydrolysis	Peptides	β-Naphthylamide hydrochloride Leucine-4-nitroaniline	Brown (1985); Burket and Dick (1998); Marx et al. (2001); Vepsäläinen et al. (2001); Stemmer (2004)
Leucine-alanine			L-Leucine 7-AMC*	
Arginine-alanine			L-Arginine 7-AMC	
Tyrosine-alanine			L-Tyrosine 7-AMC	
Lysine-alanine aminopeptidase			Lys-ala 7-AMC	
Denitrification activity (DEA)	Denitrification of nitric compounds to N_2O and N_2	Nitric compounds	Nitrates	Luo et al. (1996); Simek and Hopkins (1999); Simek et al. (2002)
L-Asparaginase (EC 3.5.1.1)	Hydrolysis of asparagine to aspartate and NH_3	L-Asparagine	L-Asparagine	Frankenberger and Tabatabai (1991a); Dodor and Tabatabai (2003)

Table 12.1. (continued)

Enzyme (trivial name)	Function	Natural substrates	Substrates used in the assay	References
L-Aspartase (EC 4.3.1.1)	Hydrolysis of L-aspartate to fumarate and NH_3	L-Aspartic acid	L-Aspartic acid	Senwo and Tabatabai (1999); Dodor and Tabatabai (2003)
L-Glutaminase (EC 3.5.1.2)	Hydrolysis of glutamine to glutamate and NH_3	L-Glutamine	L-Glutamine	Frankenberger and Tabatabai (1991b); Dodor and Tabatabai (2003)
L-Glutamine synthetase (EC 6.3.1.2)	Reaction between NH_4^+-N and L-glutamic acid	L-glutamic acid and NH_4^+-N	NH_4^+-salts and nitrates	McCarty (1995); Landi et al. (1999)
L-Histidase (EC 4.3.1.3)	Hydrolysis of hystidine to urocanate and NH_3	L-Histidine	L-Histidine	Frankenberger (1983); Burton and McGill (1989, 1991)
Nitrate reductase	Reduction of NO_3^- to NO_2^-	Nitrates	KNO_3	Abdelmagid and Tabatabai (1987); Fu and Tabatabai (1989)
Nitrogenase	N_2 fixation	Molecular nitrogen	C_2H_2	Tann and Skujins (1985); Martinez-Toledo et al. (1988); Gajendiran and Mahadevan (1989); Halsall and Gibson (1991)
Protease	Protein hydrolysis	Natural and added proteins	Casein, *N*-benzoyl-L-argininamide (BAA), *N*-benzyloxy-carbonyl-L-phenylalanyl L-leucine (ZPL)	Bonmati et al. (1998); Gianfreda and Bollag (1996); Nannipieri et al. (2002); Tabatabai and Dick (2002)

Table 12.1. (continued)

Enzyme (trivial name)	Function	Natural substrates	Substrates used in the assay	References
Rhodanese	Reaction between cyanide and thiosulfate to produce thyocianate	Transformation of sulfur organically bound compounds	Thiosulfate and cyanide	Tabatabai and Singh (1976, 1979); Singh and Tabatabai (1978); Ray et al. (1985); Szadjach (1996)
Urease	Hydrolysis of urea into ammonium and carbon oxide	Urea	Urea	Bremner and Mulvaney (1978); O'Toole (1991); Kandeler et al. (1999a); Klose and Tabatabai (1999a); Sinsabaugh et al. (2000); Taylor et al. (2002)

*7-AMC = 7-amino-4-methylcoumarin

enzymes are not extracted from soil seems to prove the low importance of this enzymatic category in soil.

Another aspect of the complexity of enzyme activities in soil is their in situ distribution, i.e. their spatial variability in a soil profile and their localisation in soil structural fractions of different nature and size. As reviewed by Gianfreda and Bollag (1996), earlier studies on this matter were performed by Ross and Speir and their co-workers and by Perez-Mateos and his collaborators.

Bergstrom and Monreal (1998) and Bergstrom et al. (1998b, 2000) found that arylsulfatase activity was spatially decreased with depth and water content along a slope of the Ap horizon in a Gray Brown Luvisol (Hapludalf), sampled on 74 positions following harvest of soybean (*Glycine max* L. Merr.) and fall tillage. Phosphatase activity spatially depended on organic C and inorganic P contents whereas dehydrogenase, urease, glutaminase and β-glucosidase activities showed little or no spatial dependence. The six enzyme activities were also measured in a Rego Humic Gleysol (Aquoll) sample (0–23 cm depth) strip-cropped to corn (*Zea m*ays L.) and soybean (*Glycine max* L. Merr.) and these samples were collected at 20 cm spacing along transects across adjacent plant rows (Bergstrom and Monreal 1998). Enzymatic activities of soil samples collected from within crop rows were compared with those of samples collected between crop rows. The spatial pattern of all six soil enzyme activities was sometimes influenced by the crop and they behaved similarly as general indices of microbial activity at the scale of measurement.

Generally, enzyme activities decline with depth and are related to microbial activities and C and N organic contents of soil (see Sect. 12.7).

Kandeler and co-workers have emphasised the importance of studies on the micro-scale distribution of enzyme activities and soil microorganisms in particle-size soil fractions and their variations at a small-scale level in response to different agricultural practices (i.e. organic amendments, tillage; Stemmer et al. 1998, 1999; Kandeler et al. 1999a,b,d; Gerzabek et al. 2002; Poll et al. 2003). These studies can be useful: (1) to study the importance of different soil fractions on the protection of microorganisms and enzyme activities associated with organo-mineral particles (Kandeler et al. 1999d); (2) to elucidate if and how the metabolic activities are distributed in different particle size fractions (Gerzabek et al. 2002); and (3) to evaluate the contribution of different particle size fractions to the turnover of organic C, by following the response of microbial processes to a range of long- and short-term organic C inputs (Stemmer et al. 1999; Gerzabek et al. 2002; Poll et al. 2003).

The poor knowledge about the location of enzymes in differently sized particle fractions may have been due to the procedures used for physical fractionation in the past, which greatly reduced both soil microbial

biomass and enzyme activities (Ruggiero et al. 1996). It was concluded that soil microorganisms were mainly associated with the silt and clay fraction (Ladd et al. 1996). In recent investigations, a more preservative fractionation method preceded by low-energy sonication of soil (because it minimised destruction of labile particulate organic matter) was adopted (Stemmer et al. 1998, 1999; Kandeler et al. 1999a,b,c; Gerzabek et al. 2002; Poll et al. 2003).

The distribution of enzyme activities between bulk and rhizosphere soil and the accumulation of enzymes at soil-plant root interfaces have been studied (Tarafdar and Jungk 1987; Badalucco and Kuikman 2001; Naseby and Lynch 2002). A high activity of arylsulfatase per unit of microbial biomass-S was observed in rape rhizosphere soil, and organic acids were considered the most efficient substrates in promoting the production of the enzyme by the soil microflora (Vong et al. 2003). The acid phosphatase and dehydrogenase activities of rhizosphere soils from 13 nodulated legume species sampled at two savanna sites with very different chemical properties were significantly higher than the respective enzyme activities of bulk soils (Izaguirre-Mayoral et al. 2002). Differences among species and between sites were recorded.

Although root-associated microorganisms often are assumed to be the main producers of enzymes in the rhizosphere soil, an important contribution of enzymes can, however, derive from enzymatic proteins produced by plant roots and released in their surrounding rhizosphere soil. These enzymes are usually wall-associated enzymes and catalyse the formation of products which are taken up by plant roots or rhizosphere microorganisms (Gramss et al. 1999b; Chroma et al. 2002; Harvey et al. 2002).

The enzymes typically more abundant in the rhizosphere are phosphatases whose activities are related to the depletion of organic P (Tarafdar and Jungk 1987). Furthermore, a strict correlation between phosphatases originating from plant roots and P nutrition of plants was demonstrated, particularly in rhizosphere soils of mycorrhiza-infected plants (Halstead and McKercher 1975; Dick et al. 1983; Chhonkar and Tarafdar 1984; Reddy et al. 1987; Tarafdar and Jungk 1987; Tarafdar and Claassen 1988; Haussling and Marschner 1989; Tadano and Sakai 1991; Fox and Comerford 1992; Tarafdar and Marschner 1994). The localisation of phosphatase activity in rhizosphere soil was carried out by microscopic observations of soil sections, after their treatment with suitable compounds, which allowed enzymes in soil structure to be localised, or reaction with substrates giving rise to products easily visualisable as coloured compounds (Ladd et al. 1996; Joner et al. 2000).

Grierson and Adams (2000) concluded that seasonal and spatial heterogeneity of acid phosphatase activity of rhizosphere soil depended on plant species composition and root type (cluster roots or ectomycorrhizal

roots), in a study comparing the enzyme activity, ergosterol (as indicator of fungal biomass) and microbial P in soils from a forest of south-western Australia dominated by Jarrah (*Eucalyptus marginata* Donn ex SM.) with and without *Banksia grandis* Wiild. Jarrah is characterised by an extensive surface system of fine lateral roots with ectomycorrhizal associations, while *Banksia grandis* is the producer of a mat of cluster (proteoid) roots and is capable of growing if fire is excluded from these forests. Increases in acid phosphatase activity were associated to increases in microbial P and ergosterol concentrations of soils and, thus, it was concluded that most of the measured enzyme activity was due to fungal activity.

Further evidence on the effect of plant species on enzyme activities of rhizosphere soil were provided by Gramss et al. (1999a) who demonstrated that several members of *Fabaceae*, *Gramineae* and *Solanaceae* released considerable amounts of peroxidase, laccase, monophenol monoxygenase, and proteinase-lipase-esterase activity, expressed as fluorescein diacetate hydrolase, in the rhizosphere of both sterile and non-sterile soils. Such enzyme activities were lower in non-sterile than sterile soils probably because they were inactivated by proteases of soil microorganisms. The protein preparations from planted soils contained plant peroxidase as evidenced by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analyses and isolectrofocusing (Gramss et al. 1999a). The production of cell-wall-associated peroxidase was also demonstrated in vitro in some cultures of plants of various species and morphology (Chroma et al. 2002; Harvey et al. 2002).

The capability of a soil to show a catalytic and enzymatic activity, even in the absence of any living cells, led to the conclusion that active enzymatic proteins must act in soil as stable complexes with inorganic and organic soil colloids. As defined by Gianfreda and Bollag (1996), these enzymes can be regarded as "naturally immobilised enzymes".

Two main features differentiate a soil enzyme from its pure preparation: the kinetic behaviour and the sensitivity to various denaturing agents. Soil enzymes usually have lower V_{max} values and higher K_m constants, indicating a lower catalytic efficiency and a reduced substrate affinity, than the respective purified enzymes from different sources (Nannipieri and Gianfreda 1998). Similarly, the extensive bibliography shows different activity-pH or activity-temperature profiles (Nannipieri and Gianfreda 1998). These peculiar behaviours can partially be explained by assuming that soil enzymes are immobilised on soil supports and, consequently, work in a heterogeneous system (McLaren and Packer 1970; McLaren 1975, 1978; Nannipieri and Gianfreda 1998). However, the exact mechanism by which enzymes are immobilised and consequently stabilised in soils is not completely clarified.

Numerous studies have examined the adsorption of proteins on clay minerals and the properties of the resulting clay–protein complexes, because the clay fraction can play an important role in the immobilisation of soil enzymes (Kiss et al. 1975; Theng 1979; Boyd and Mortland 1990). This topic is discussed in Chap. 7. On the other hand, experimental evidence obtained on the interactions between proteins and organic substances indicates that soil organic matter can also have an important role in the immobilisation of enzymes in soil (Ladd and Butler 1975). Lähdesmäki and Piispapen (1992) concluded that the extraction, the fractionation by gel filtration and the dilution damaged or destabilised the protective mechanism (against environmental stresses in soil) by soil colloids on protease, cellulose and amylase.

The main strategy used for understanding the relationships between immobilised enzymes and their clay or humic supports was to study properties and behaviours of enzyme complexes, obtained in vitro by the interaction between enzymes and clay minerals, humic substances or organo-mineral complexes (Ladd and Butler 1975; Burns 1978a; Boyd and Mortland 1990; Gianfreda et al. 2002). The support, the enzyme, the bond between enzyme and support, the chemical or physical mechanism implicated in the immobilisation process are all important in the formation of the complex and markedly affect the properties of the resulting complex. Cation-exchange adsorption mechanisms, van der Waals type forces, ionic or hydrophobic bonds, can hold the enzymes to clay surfaces. Ion exchange, entrapment within organic networks, ionic or covalent bonding, may account for the stable association between enzymes and humic materials (Ladd and Butler 1975; McLaren 1975; Burns 1978a, 1982, 1986; Theng 1979; Boyd and Mortland 1990).

Enzyme activities in soil are resistant to proteolysis, thermal and chemical denaturation (Nannipieri et al. 1996). To achieve an increased stability, enzymatic proteins must preserve not only their three-dimensional structure or conformation, but also their plasticity to explicate their catalytic action. Several mechanisms may contribute to enhance enzyme stability upon immobilisation on solid supports: the tightening of the protein structure by covalent (mono or multipoint) bindings to an insoluble support or by "cage effects" inside a polymeric gel; the change of the enzyme microenvironment; the steric hindrance created by the surrounding matrices with reduced molecular mobility and capacity of unfolding; an increased physical protection towards proteases. The presence of special molecules such as sugars, polyalcohols and organic polymers or of an organic phase can also lead to increased enzyme stability (Gianfreda and Scarfì 1991). These mechanisms and conditions can also be valid for soil enzymes. The physical and chemical conditions surrounding enzymes immobilised by clay minerals, entrapped in the humic matrix,

biological membranes or by particular subcellular structures, are different from those of aqueous solutions. Thus the kinetic characterisation of these immobilised enzymes has to take into consideration the heterogeneous phase in which they carry out their activity (Nannipieri and Gianfreda 1998).

12.3 Factors Affecting Soil Enzyme Activities

Due to the complexity of the soil environment it seems evident that several factors will affect enzyme activities in soil. As discussed by Gianfreda and Bollag (1996) and Dick and Tabatabai (1999), the activity of any enzyme in soil is the result of synthesis, persistence, stabilisation, regulation, and catalytic behaviour of the enzymatic proteins, present in the soil environment at the moment of the assay (Fig. 12.1). All these processes may be dynamically interrelated and influenced by changes in the physical, chemical, and biological composition of soil.

The expression and conservation of a soil enzyme activity may be affected by environmental natural factors (e.g. seasonal changes, different geographical locations or diverse hydric and thermal soil regimes) and

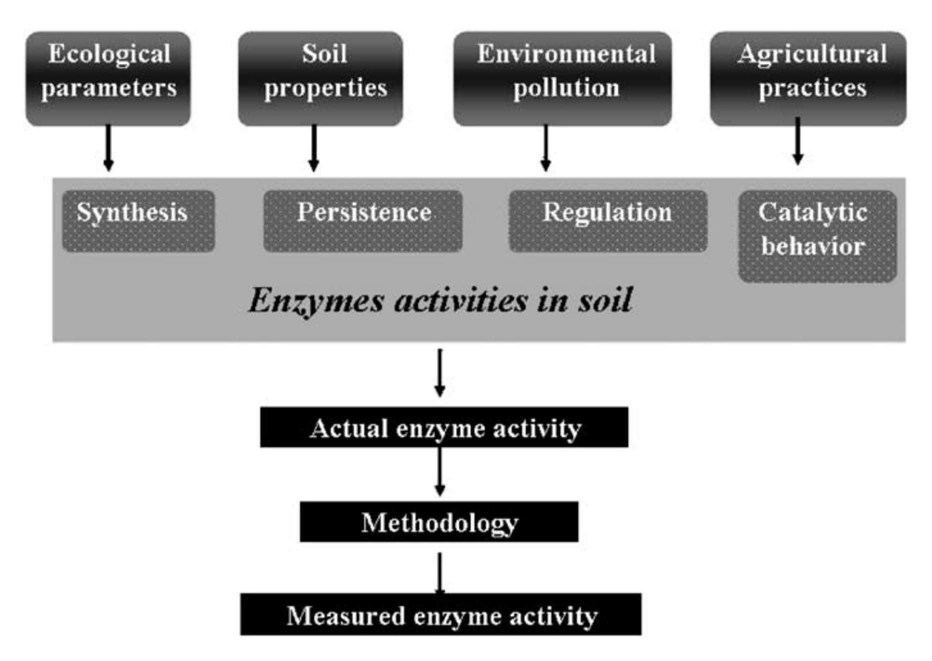


Fig. 12.1. Enzyme activities in soil (Gianfreda and Bollag 1996)

by anthropogenic activities (e.g. agriculture and management practices or pollution events). All these factors may influence the production of enzymes by plants, microorganisms, and soil biota, their persistence under natural conditions, both composition and activity of soil biota, with effect on enzyme production and release, and the immobilising and stabilising capability of soil components towards enzymatic proteins. Kiss et al. (1998) have discussed the effect of human activity on soil enzyme in a volume entitled “Enzymology of disturbed soils”.

12.4 Measurement of Soil Enzyme Activities

Soil enzyme activities have been often proposed as sensitive indicators of soil quality. As underlined by Schloter et al. (2003), “the ideal soil microbiological and biochemical indicator to determine soil quality would be simple to measure, should work equally well in all environments and reliably reveal which problems existed where”. The authors considered enzymatic assays very helpful methodologies because they are short-term laboratory procedures, usually performed under standardised environmental conditions (use of sieved samples, optimal temperature and pH, and standardised water content), which prevent or minimise changes in the composition of soil biota and allow comparison of data from different soils obtained in different laboratories.

However, accurate and reliable methodologies are required to understand the cause-effect relationship between a considered parameter and an observed soil enzyme activity modification. Two different types of problems arise when measuring the enzyme activity of soil. The first concerns the selection of enzymes to be measured and mostly depends on the objectives of the research being conducted. The second problem relates to the intrinsic nature of the soil enzyme to be measured and concerns the selection of the proper method. However, it is also important to keep in mind that enzyme-like reactions occur in soil and may contribute to the measured enzyme activity (Ruggiero et al. 1996).

If the goal of the research is to characterise different soils for their microbial activity and/or to derive indices of soil microbial functional diversity, the activities of a great number of enzymes should be measured. Indeed, a single measure very unlikely could represent an indicator of the functional diversity, which reflects a multitude of biochemical pathways (Nannipieri et al. 1990; Schloter et al. 2003). Furthermore, attention should be paid to enzymatic activities of the main metabolic pathways, such as dehydrogenase or assimilatory nitrate reductase activities which are active in living cells (Dick 1994).

If the goal of the research is focused on process-level investigations such as response of soil to the application of fertilisers, amendments or pesticides, evaluation of land-use impacts, or monitoring soil pollution and recovery upon remediation actions, the measurement of enzyme activities involved in the transformation of specific substrates, without specific attention to their origin or location, may be suitable. Also in this case, the assay of a single enzyme activity is not sufficient for the reasons discussed above.

Enzyme assays are based on the quantitative evaluation of the product(s) released or of the substrate(s) consumed and thus they do not distinguish the contribution of the different enzyme categories to the overall enzyme activity (Burns 1982). As discussed above, the same enzyme can be intracellular in living cells, in dead cells or cell debris or extracellular, free or absorbed by soil colloids. By simplifying we can say that the enzyme can exist in soil, for example, in three forms: free in solution, inside a living cell and immobilised on an organo-mineral complex. It is obvious that these enzyme forms can behave in a different way. For example, the immobilised form can be very stable, and preserve its activity almost completely, whereas the activities of the other two forms can be inhibited by an inhibitor.

Nannipieri et al. (2002) have critically and thoroughly examined the different methodological approaches to distinguish among the different categories of soil enzymes. Particular attention has been paid to the identification and quantification of the intracellular and extracellular fractions contributing to the overall soil enzyme activity. This is definitely one of the most important problems in the interpretation of soil enzymatic activities. One method is based on the measurement of microbial growth and enzyme activity during incubation when soil is amended with C and N substrates. Each enzyme activity is plotted against microbial biomass, measured at the same incubation time; if a linear correlation exists, the extrapolation of the straight line to zero microbial biomass gives the intercept of the plot on the ordinate (enzyme activity) which represents the extracellular enzyme activity in case of a positive value. This approach assumes that enzyme assays detect both intracellular and extracellular enzyme activity. The second method assumes that enzyme measurements only detect extracellular enzyme activity (Klose and Tabatabai 2002). After $CHCl_3$ fumigation or ultrasonic treatment it is possible to measure both intracellular and extracellular enzyme activity due to the lysis of microbial cells. This method presents the drawbacks that proteases are active during $CHCl_3$ fumigation and partially hydrolyse enzymes (Renella et al. 2002) and released enzymes can be adsorbed by soil colloids with consequent denaturation (Fornasier 2002). These processes can obviously lead to an underestimation of the contribution of intracellular enzymes. Furthermore, the efficacy of $CHCl_3$ fumigation on the lysis of microbial cells may change with changing soil

structural properties, thus affecting the reliability of the method (Badalucco et al. 1997).

The measurement of the enzyme activities of soil before and after ultrasonication has also been proposed as a reliable method to distinguish between intra- and extracellular enzyme activities in soil (Badalucco, pers. comm.). Apart from the same problems of the $CHCl_3$ fumigation method, the ultrasonic treatment of soil can facilitate the disaggregation and fractionation of mineral particles and organic matter, thus favouring the possible release of enzymes associated to soil colloids and whose activity could not be determined before the ultrasonic treatments. Indeed the consistent increase in acid phosphatase activity (up to 156% greater than the untreated sample) has been hypothesised to be due to ultrasonication with release of a large portion of acid phosphatase activity, undetected when associated to soil colloids (De Cesare et al. 2000). However, it was concluded that the release of intracellular acid phosphatase after lysis of cells upon sonication could not be excluded.

Different chemical agents have also been added to the enzyme assays during the incubation to inhibit the microbial growth and the consequent increases in enzyme activity due to new synthesis of proteins. Toluene is one of the most used antiseptic agents in enzyme assays, especially in the hydrolases assays, but it presents some disadvantages such as direct inhibitory or stimulatory effects on the activity of the tested enzymes (Acosta-Martinez and Tabatabai 2002) or the increase of microbial cell permeability with consequent overestimation of the intracellular enzyme activity (Skujins 1978; Nannipieri et al. 2002). However, Klose and Tabatabai (1999a,b) demonstrated that both intracellular arylsulfatase or urease activities were not completely detected upon toluene treatment and that the enzymes were not inhibited by toluene.

A reasonable approach to determine the so-called stabilised extracellular enzyme activity could involve the efficient sterilisation of soil prior to the assay. Numerous methods are available to sterilise soil (Trevors 1996). However, the approach presents the following drawbacks: the method cannot be efficient in eliminating soil microbial populations; the chemical and physical properties of soil may be altered, with consequent changes in the relative fractions of immobilised enzymes; intracellular enzymes could be released upon sterilisation. Thus either an under- or overestimation of the extracellular enzyme activity of the investigated soil is possible.

The contribution of the different enzyme categories to the overall enzyme activity in soil might indirectly be evaluated by comparing properties and behaviour of enzyme activities of soils with those of synthetic enzyme model systems. This approach was followed to study the effect of three herbicides (atrazine, paraquat, glyphosate) and one insecticide (carbaryl) on invertase, urease and phosphatase activities present as: (1) free purified

enzymes, which should simulate the fractions of free enzymes in soil solution; (2) synthetic clay-, organo- and organo-clay-enzyme complexes, which should simulate enzyme-soil colloid associations; and (3) soil enzymes (Gianfreda et al. 1993, 1994, 1995, 2002; Sannino and Gianfreda 2001). Activation, inhibition or no influence depended on the pesticide and the enzyme preparation thus suggesting that the "state" of the enzyme in soil is important and no generalisations can be made. Complex responses were also obtained when the effects of the four pesticides on soil enzyme activities were studied (Gianfreda et al. 1994, 1995; Sannino and Gianfreda 2001). Increases, decreases and no effects of four pesticides on the activity of invertase, urease and phosphatase were obtained in 22 soils sampled from different sites of Italy and characterised by different physical-chemical properties (see Sect. 12.7). Furthermore, the response of some soil enzyme activities to four pesticides was similar to that of one of the model systems, probably because the model was prevalent in the relative soil. In addition, the clays of soil protected phosphatase activity against the inhibitory effect of carbaryl and atrazine. This was confirmed by the fact that phosphatase immobilised on montmorillonite was less affected by the pesticides than the free and organo- and organo-mineral-complexed phosphatase.

The conclusion of all the findings reported so far is that unfortunately methodologies capable of accurately and unambiguously measuring each of the soil enzyme components are not available to date.

In enzyme measurements, as for other microbiological and biochemical measurements, soil samples should be representative of the natural situation of studied soils (representative sampling) and the sampling strategy for biochemical and microbiological soil properties should consider their temporal and spatial dynamics (Dick et al. 1996a). Therefore, the sampling time, the number of samples and their horizontal and vertical distribution as well as the sampling procedure and device must be defined. Simple rules may be suggested in carrying out an accurate sampling procedure. To prevent temporal heterogeneity and variability, sampling should take place repeatedly during a given period of time and possibly at the same time intervals. Composite or well-mixed samples can be used and in this case a mean of the investigated soil property is obtained, provided the sampling area has uniform properties (Dick et al. 1996b). Moreover, if the aim is to obtain any information on the variability of the studied enzyme at the microsite levels, a great number of samples should be collected and geostatistical analyses should be carried out to adequately define spatial variability of the studied enzyme (Bonmati et al. 1991).

If soil pretreatment and storage are not important for measuring physical and chemical properties of soil, they are crucial in the determination of biochemical activities (Brohon et al. 1999). A short storage (maximum 10–15 days or less) of field-moist soils at 4 °C is suggested for a reliable

determination of the majority of soil enzyme activities if the measurements cannot be done immediately after sampling (Alef and Nannipieri 1995; Gianfreda and Bollag 1996; Tabatabai and Dick 2002). Changes in enzyme activities during storage depend on the period and temperature of soil storage, and the physical-chemical characteristics of the investigated soils (Brohon et al. 1999). Storage of air-dried soils has been proposed for handling soils before enzyme assay (Burns 1978a; Alef and Nannipieri 1995; Gianfreda and Bollag 1996; Tabatabai and Dick 2002), but measurements with fresh samples are more reliable (Rao et al. 2003). The response of enzyme activities to air-drying of soil as well as storage of moist soil may be enzyme-specific (Luo et al. 1996). Probably, the activities of enzymes stabilised by soil colloids are less affected than those associated to living cells by air-drying (Ladd 1985; Gianfreda and Bollag 1996). The response of enzyme activity of air-dried soils can be different in respect to that of the same enzyme activity of moist soil, thus indicating that they are different. The inhibition of several trace elements on aspartase activity (Senwo and Tabatabai 1999) was greater in air-dried than in field-moist soils whereas opposite behaviours have been observed for cellulase (Deng and Tabatabai 1995) and arylamidase activities (Acosta-Martinez and Tabatabai 2001).

Assay conditions such as presence or absence of a buffer, pH, substrates and their concentration, temperature, shaking of soil, inhibitors of microbial proliferance, etc., markedly affect the measured activity, as previously discussed (Burns 1978a; Alef and Nannipieri 1995). Usually, a soil enzyme assay is based on the use of a buffered solution of a synthetic, artificial substrate at a concentration high enough (saturation concentration) to be assumed constant throughout the time course of the enzymatic reaction and assuming a zero-order kinetics. Moreover, substrate concentration should be very much higher than that of the enzyme to allow a reaction rate proportional to the enzyme concentration.

Miller et al. (2001) suggested the use of low concentrations of substrate, so as to simulate environmental conditions. They measured two types of formamide hydrolase activity in a barley soil: a low (K_m values of 30–60 mM) and a high (K_m values of 0.5–1.0 mM) affinity enzyme activity with the latter representing the enzyme activity of active microorganisms. A similar approach was followed to measure high- and low-affinity L-glutamine synthetase (Sallis and Burns 1989) and L-histidine ammonia-lyase (Burton and McGill 1989, 1991) activities. Probably, under natural conditions, microorganisms should use high-affinity systems operating at low substrate concentration (Unanue et al. 1999).

The use of different substrates may be helpful to better understand the role of an enzyme activity in nutrient transformations in soil. For instance, Tabatabai et al. (2002) studied the activity of acyl arylamidase (an enzyme involved in one of the limiting steps of N-mineralisation) of several Iowa

soils towards eight substrates, differing in their amino acid moiety. Kinetic (K_m and V_{max}) and thermodynamic (ΔH_a and E_a) parameters depended on soil and the amino acid moiety, thus suggesting that enzyme isoforms were present in soils (Tabatabai et al. 2002).

A buffered vs. an unbuffered condition is usually chosen when the disappearance of substrate and/or the formation of reaction products may change the pH of the soil slurry, since an optimal pH value is required during the assay. Acid and alkaline soil phosphatase activities prevail in acid and alkaline soils, respectively (Eivazi and Tabatabai 1977), and thus Dick et al. (2000) proposed that acid (AcdP) and alkaline phosphatase (AlkP) activity should also be used as pH indicators.

For an assay of soil enzyme activity to be reliable and applicable, it should be tested and validated with various soils with different properties. Indeed, major problems may arise from both the adsorption of substrates and/or products on soil particles and from the possible interference by elements or compounds present in soil. For example, *p*-nitrophenyl derivatives which are common substrates in the determination of several soil enzyme (acid and alkaline phosphatases, glucosidases, galactosidases, and arylsulfatases) activities are hydrolysed to *p*-nitrophenol (pNP) which may be adsorbed by soil colloids. To avoid an incorrect evaluation of pNP-substrate-based activity, the adsorption of *p*-nitrophenol should be quantified. Thus the calibration curve should be prepared by using different amounts of *p*-nitrophenol in the presence of the soil under investigation (in the amount used in the assay) and under the experimental conditions (temperature, incubation time, addition of reagents) of the enzyme assay. Similar problems may occur when NH_4^+ is detected as a measure of urease activity in soils with different NH_4^+-fixing capabilities. Urease activity of NH_4^+-fixing and non-fixing soils was affected by the used buffer (Tris-, borate- or non-buffered; O'Toole 1991). Since small changes occur in substrate concentration under saturating substrate conditions, product appearance rather than substrate consumption is usually monitored so as to achieve higher analytical sensitivity.

Some compounds or elements of soils under investigation may react with the reaction products thus affecting the measured activity. For example, Cu reacts abiotically with triphenylformazan, the end product of dehydrogenase activity measured by using tetrazolium salt (TTC) as acceptor of electrons (Chander and Brookes 1991). Unfortunately, Cu also reacts with 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium formazan (INTF), the end product of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT), the alternative substrate used to measure the dehydrogenase activity of soil (Obbard 2001). Paraquat affects the *p*-nitrophenol determination at 400/405 nm in alkaline environment (Gianfreda et al. 1993). Coloured compounds extracted from soil with some buffers can affect the colorimetric determination of substrates or reaction products.

The use of substrate derivatives, with enzymatic production of fluorescent end products, has been proposed as a valid alternative to increase the sensitivity of the enzymatic assays and to avoid some of the problems cited above (Marx et al. 2001). These assays are based on the use of fluorophore-labelled artificial substrates, derived from water-soluble fluorophores (e.g. hydroxy- and amino-substituted coumarins, fluorescein, and rhodamine), which are enzymatically transformed into highly fluorescent, water-soluble products with optical properties different from those of the parent compound. The enzyme assay is rapid, can be used to measure several enzyme activities of soil with the microplate approach, and it allows the measurement of several soil samples (Wirth 1992; Wirth and Wolf 1992; Sinsabaugh et al. 2000; Marx et al. 2001; Vepsäläinen et al. 2001; Stemmer 2004; Wittmann et al. 2004). The advantages and disadvantages of the microplate fluorimetric assay and the *p*-nitrophenol method are summarised in Table 12.2.

Although the multiple-substrate approach provided results closely correlated to those obtained with the classical enzyme assays, it presented some drawbacks that may limit its application to different soils (Stemmer 2004). Indeed, the substrate recovery was incomplete because of its adsorption onto the soil matrix, soils with different physical and chemical properties behaved differently, competitive inhibition occurred due to chemically similar substrates, and data from soils with contrasting pH values could not be compared because the multiple-substrate assay was conducted at soil pH (Stemmer 2004).

As has been extensively discussed, the present enzyme assays give potential rather than real enzyme activities due to experimental conditions adopted in the determination (Burns 1978a, 1982; Nannipieri et al. 1990, 2002; Gianfreda and Bollag 1996). For this reason, Schloter et al. (2003) have suggested that the present soil enzyme assays give a measure of the enzyme activity "encoded in the soil genotype" and not a "phenotypic activity". This obviously restricts the usefulness of enzymatic activity data and limits their interpretation.

Another problem is the lack of standardisation because different protocols are used to determine an enzyme activity in soil, as indicated by Burns (1978b) for assaying urease activity. Unfortunately, this variability still continues, as shown in Table 12.3, which reports the experimental conditions used for assaying soil urease in the last 5 years. It is worth noting that different conditions have been adopted by the same research groups in different studies and no reasonable explanation of such variability is provided.

Another problem is the presence of enzyme-like reactions which cannot be distinguished by enzyme reactions with the present assays (Huang 1995; Ruggiero et al. 1996; Nannipieri et al. 2002). Clays, metal oxides and hydroxides can catalyse a large number of reactions; e.g. organic

Table 12.2. Comparison between the 4-methylumbelliferone (MUB) micro-plate fluorimetric assay and the *p*-nitrophenol assay for measuring soil enzyme activity (from Marx et al. 2001)

4-Methylumbelliferone	*p*-Nitrophenol
The products	
Measured fluorimetrically	Measured colorimetrically
Excitation at 365 and emission at 460 nm	Absorbance at 405 nm
MUB fluorescence is pH-dependent	*p*-Nitrophenol absorbance is pH-dependent
Highly sensitive especially at low concentrations	Low sensitivity
No known side effects	
Quenching of fluorescence by soil particles and phenolic compounds	*p*-Nitrophenol interferes with organic material
MUB molecule is highly mobile	*p*-Nitrophenol is adsorbed
The assay	
Soil added volumetrically	Soil added gravimetrically
Small quantity of substrate needed (μl)	Large amounts of substrate required (ml)
Short incubation time (35 min)	Medium to long incubation time (1–24 h)
Substrate difficult to dissolve	Substrates dissolve instantaneously
Continuous monitoring of product release over time (1 read cycle min^{-1})	A single reading at the end of the incubation
Measurement directly carried out in the reaction medium	Need to stop the reaction and extract the product prior to measurement (measurement must not be delayed, otherwise there is formation of Na_2CO_3)
High numbers of samples and replicates processed at the same time (96-well $plate^{-1}$)	Maximum of 50 assays per run
Plate set-up and measurement in less than 1 h	Assay preparation, incubation, product extraction and measurement >3.5 h
Disposable materials (micro-plates, universal bottles, pipette tips)	Use of glass equipment with time-consuming preparation
Results	
Automatic calculation of activity rates (relative units of fluorescence min^{-1})	Withdrawal of background absorbance
Miscellaneous	
Expensive analytical equipment [fluorimetric plate-reader, multichannel (digital) pipettes, micro-plates]	Analysis at low cost
A limited number of potential substrates available	A large number of substrates available

compounds, usually of phenolic nature, are transformed in their oxidised products, and these abiotic reactions are involved in the humus formation (Ruggiero et al. 1996).

Table 12.3. Experimental conditions used in soil urease assays

Parameter	Klose and Tabatabai (1999a)	Sinsabaugh et al. (2000)	Dilly and Nannipieri (2001)	Taylor et al. (2002)	Caravaca and Roldán (2003)	Renella et al. (2004)
Urea concentration	0.20 M	0.02 M	0.08 M	8.9 mM	1 M	Not specified
pH	9.0	5.0	Not specified	10	7.0	Not specified
Buffer	0.1 M THAM	50 mM acetate (or deionised H_2O)	Not specified	75 mM borate buffer	0.1 M phosphate	0.1 M phosphate
Incubation temperature	37 °C	20 °C	37 °C	20 °C	30 °C	37 °C
Incubation time	2 h	18 h	2 h	4 h	1.5 h	1 h
Soil/aqueous solution ratio (w/v)	Not specified	1:100	2:1	5:22.5	0.5:0.5	0.5:2
NH_4^+ measurement	Not specified	Salicylate-cyanurate reagent kit	Salicylate-cyanurate reagent kit	Salicylate-cyanurate reagent kit		Flow injection analyser coupled with a spectrophotometer
Soil status, treatment or pre-treatment	Field-moist soil	Soil homogenisation in acetate buffer pH 5	Fresh soil	Field-moist soil stored at 4 °C	Field-moist soil stored at 4 °C	Soil moistening to 50% WHC and preincubation for 7 days at 25 °C

12.5 Soil Functioning as Determined by Enzyme Activity

Since taxonomic diversity of microorganisms and environmental factors determines the actual enzyme processes in soil (Kandeler et al. 1996), it has been suggested that soil enzyme measurements can be useful and sensitive indicators of variations in soil functions and activity. However, as discussed before, one or a few enzyme activities cannot be taken as indicator of a complex metabolic picture such as soil functioning, and present enzyme assays give potential rather than actual enzyme activity.

12.5.1 Degradation of Litter and Enzyme Activities in Forest Soil

Since the 1980s, the dependence of the soil enzyme activities on vegetation and decomposition of fresh plant residues has been emphasised (Ross 1976; Spalding 1977). Duxbury and Tate (1981) claimed that the variations in the activities of acid phosphatase, invertase, cellulase, xylanase, amylase, and polyphenol oxidase caused by soil depth and differences in crop were greater than those caused by season variability. In some soils, microbial enzymes have been suggested to prevail over plant enzymes. For example, alkaline phosphatase activity of microbial origin was prevailing over acid phosphatase activity synthesised by plants and fungi (Naseby and Lynch 1998; Acosta-Martinez and Tabatabai 2000; Sect. 12.2).

The role of enzyme activities involved in litter decomposition has recently been reviewed by Sinsabaugh et al. (2002a). Multiple enzyme systems are collectively involved in a synergistic interaction. The decomposition process is seen as a "successional loop" where: "the substrate selects the microbial community, which produces extracellular enzymes that degrade and modify the substrate, which in turn, drives community succession. In this model, extracellular enzymes link substrate composition and microbial community metabolism". Indeed, polymer changes in plant litter composition produced differences in decomposition patterns. Xylanase and invertase activities of the decaying green materials were initially low, when decomposition of water-soluble substrates was predominant, but these enzyme activities increased over time, indicating a possible relationship between invertase and xylanase, in which the reaction catalysed by xylanase furnished substrate for invertase. In contrast, the decomposition of brown materials, containing low amounts of soluble substances and high lignin, occurred within a rigid framework, where the number of sites for enzymatic attack remained constant over time (Luxhøi et al. 2002).

Nitrogen deposition can affect litter decomposition rates by affecting the release of extracellular enzyme activities by the forest-floor microbial community. During the degradation of three types of deciduous leaf litter, cellulase activity was increased by N deposition, but the activity of ligninolytic enzymes produced by white-rot fungi declined substantially for lignified red oak (Sinsabaugh et al. 2002b). Glycosidase activities, associated with the decomposition of cellulose, chitin, and storage carbohydrates, were generally enhanced by N amendments in both litter and soil of an *Acer saccharum* forest (Saiya-Cork et al. 2002). In contrast, addition of mineral N to organic layer material of various Norway spruce sites affected the various cellulase activities, but not in a uniform way (Michel and Matzner 2003), because endoglucanase activity increased in N-treated samples, whereas β-glucosidase activity decreased, most markedly in the presence of litter with wider C-to-N ratios (Saiya-Cork et al. 2002). Other experiments have confirmed the suppression of the ligninolytic activity and the decrease of decay rates of plant matter in lignified litter by N addition (Carreiro et al. 2000). Phenol oxidase activity was repressed by N amendment in soil but increased in litter and the mass loss rates from litter were significantly correlated with phenol oxidase activity (Carreiro et al. 2000; Saiya-Cork et al. 2002; Sinsabaugh et al. 2002b). No suppressive effects of mineral N on polyphenol oxidase and N-acetylglucosaminidase have also been recorded by Michel and Matzner (2003). Enzymes involved in N reactions, such as urease, β-acetylglucosaminidase, and leucine aminopeptidase, showed mixed responses depending on N availability, as affected by N fertilisation (Saiya-Cork et al. 2002). Sinsabaugh et al. (2002b) concluded that N amendment reduced enzyme efficiencies, changed extracellular enzyme activity with a shift from enzymes involved in N acquisition to enzymes involved in P acquisition, and from those involved in polyphenol oxidation to those involved in polysaccharide hydrolysis.

Larson et al. (2002) studied the relationship between changes in chemical composition of plant litter under elevated carbon dioxide and several enzyme activities. The greater plant growth under elevated CO_2 significantly increased the activities of the enzymes involved in the cellulose and chitin degradation. In contrast, elevated ozone concentration counteracted the CO_2 effect on cellobiohydrolase activity.

The interpretation of measurement of enzyme activities in forest soils must consider the peculiarities of the investigated system, such as types of organic horizons and litter, quantities and qualities of humic substances, climate, pH, and depth of sampling. It may be concluded that enzyme activities of forest soils are more sensitive to season and soil horizon than to changes in forest cover (Criquet et al. 2000; Kourtev et al. 2002; Papa et al. 2002; Wittmann et al. 2004).

12.5.2 Effect of pH

Each enzyme exhibits a characteristic pH profile, an optimum pH for its maximum activity and a specific stability which is related to soil pH (Gianfreda and Bollag 1996). Generally, enzyme activities of soils often increase by increasing soil pH with the exception of acid phosphatase which is predominant in acid soils (Deng and Tabatabai 1997; Acosta-Martinez and Tabatabai 2000; Ekenler and Tabatabai 2003a,b).

It is worth underlining that when the effects of soil pH on enzyme activities are studied, the concentration of organic matter and organic and inorganic N should remain constant (Acosta-Martinez and Tabatabai 2000; Ekenler and Tabatabai 2003a,b). For example, the significantly greater activities of alkaline phosphomonoesterase, inorganic pyrophosphatase, and phosphodiesterase in manure-treated soils were not only due to greater soil pH values, but also to enhanced microbial activity and diversity due to manure input over the years (Parham et al. 2002).

Liming and the consequent pH change can affect the distribution in soil of fungal and bacterial biomass (Baath et al. 1992) and thus patterns and activities of enzymes. Liming applications increased the activities of many enzymes in long-term management soils indicating a positive correlation between enzyme activities (with the exception of acid phosphatase) and soil pH (Acosta-Martinez and Tabatabai 2000; Ekenler and Tabatabai 2003a,b). An order of enzyme sensitivity (expressed as Δactivity/ΔpH ratio values) was established, with L-glutaminase activity being the most sensitive and L-aspartase activity being the least sensitive enzyme activity (Acosta-Martinez and Tabatabai 2000).

The pH of soil can be markedly reduced after sludge application, particularly in the light textured sandy soil with a low buffering capacity, due to nitrification of the NH_4^+-N produced by mineralisation of added organic matter and sulfide oxidation. The decrease in soil pH causes a solubilisation of heavy metals with possible adverse effects on enzyme activities. Liming of these soils raises soil pH values and alleviates the toxicity of metals.

12.5.3 Effect of Stresses

Salinity

The potential adverse effects of the accumulation in soil of soluble salts on the activities of soil enzymes were studied in laboratory experiments (Frankenberger and Bingham 1982) also involving gypsiferous soils (García and Hernández 1997). Many enzyme activities were reduced with

increasing electrical conductivity of saturated extracts and the degree of inhibition varied among the enzymes assayed and the nature and amounts of salts added. More recently, a different approach has been adopted, involving the adverse effects of irrigation-induced salinity and sodicity on soil enzyme activities (Rietz and Haynes 2003). Fluorescein diacetate (FDA) hydrolysis and the activity of β-glucosidase, alkaline phosphatase and arylsulfatase declined exponentially by increasing salinity and linearly by increasing sodicity, as the result of denaturation of enzyme proteins at high salt concentrations and decrease in microbial biomass and activity. However, even at high electrical conductivity and sodium adsorption ratio (SAR) values, substantial enzyme activity can persist, likely due to the enzyme production by a bacterial flora adapted to saline soil conditions (Zahran 1997).

Heavy Metals and Pesticides

Generally speaking, the effects of a particular organic toxicant are temporary and depend on the ability of the microorganisms to adapt to its presence and to degrade it. In contrast, toxic metals can remain in soil and cause long-term damage to soil microbial communities, and can negatively affect several biotic and abiotic processes (Adriano 1988; Dungan and Frankenberger 2002). Heavy metals affect enzyme activity by modifying the protein conformation, due to interaction with the protein active groups and by inhibiting enzyme synthesis (Nannipieri 1994; Gianfreda and Bollag 1996; Ruggiero et al. 1996). However, indirect effects are also possible because changes in microbial communities of soil can modify the enzyme activity (Nannipieri 1994). Indirect effects on sulfatase activity, respiration and substrate-induced respiration (SIR) response were due to soil acidification following the addition of heavy metal nitrate salts (Speir et al. 1999). Thus it is obvious that effects of heavy metals on soil enzyme activities depend on the tested enzyme, soil type, the heavy metal and the compounds added to soil with the metal.

Kandeler et al. (1996) showed that cellulase, xylanase and β-glucosidase activities did not change significantly at the lowest heavy metal concentration whereas arylsulfatase and phosphatase activities were markedly affected.

Twenty-five trace elements inhibited more cellulase activity of field-moist than air-dried soils (Deng and Tabatabai 1995). Eighteen of the 25 trace elements inhibited arylamidase activity in both air-dried and field-moist samples, five metals activated this enzyme activity regardless of the soil moisture content, and two metals activated arylamidase activity in air-dried soils, but inhibited it in the field-moist soils (Acosta-Martínez and Tabatabai 2001). Eighteen of 23 trace elements inhibited the activity

of β-glucosaminidase and five activated the enzyme activity. Since the enzyme was assayed after 1 h of equilibration of the soil-trace element solution mixture, variations in soil physicochemical processes other than interactions of metals with enzyme proteins could be responsible for the differences observed (Ekenler and Tabatabai 2002b).

Of course the inhibitory effect of heavy metals on enzyme activity can be masked by associated sewage sludge, which can stimulate microbial activity and thus enzyme activity (Nannipieri 1994).

Both dehydrogenase and nitrate reductase activities were inhibited by selenic acid after 112 days of incubation because probably selenium replaced sulfur in the active sites of the enzymes whereas β-glucosidase activity was not affected (Nowak et al. 2002).

Little information is available on the long-term effects of heavy metal pollution on soil enzyme activities under field conditions probably because a control soil accurately representing soil in its pristine state is rarely available (Giller et al. 1998). Arylsulfatase, phosphatase, urease and xylanase activities decreased in particle-size fractions after 10 years of contamination with different levels of Zn, Cu, Ni, V, and Cd (Kandeler et al. 2000). It was hypothesised that the decrease in arylsulfatase and phosphatase activities was caused by a reduced enzyme synthesis by soil microorganisms due to heavy metals, rather than by a direct inhibition on the enzyme activities. In addition, both enzyme activities and microbial biomass were more sensitive to heavy metals than bacterial diversity assessed by denaturing gradient gel electrophoresis (DGGE).

Regardless of cultivation (maize or grass) or set-aside regime, alkaline phosphomonoesterase, arylsulfatase and protease activities were significantly reduced in soils contaminated with Cd for about 15 years, whereas acid phosphomonoesterase, β-glucosidase and urease activities remained unaffected (Renella et al. 2004). Also in this case bacterial community was not affected.

The bibliography about the effects of pesticides on soil enzyme activities is extensive (Schäffer 1993). These effects depend on the type of enzyme, soil properties, experimental procedure and type and rate of pesticide application. Partition, adsorption, and immobilisation of pesticides in soils are processes that directly affect pesticide–enzyme interactions. The pesticides can directly bind to extracellular enzymes in reversible and irreversible mode altering the protein conformation and its activity. The same effect may occur by the degradation products after the parent substrate has been degraded. Indirect effects are mainly assigned to pesticide-induced changes in size, structure and functionality of the microbial community. If recommended field application rates are used, inhibitory effects are temporary, and enzyme activities return to levels similar to those of untreated soils after a few weeks or months (Ladd 1985). However, due to this complex

picture, it is obvious that many results are conflicting, and it is not reasonable to compare laboratory and field studies and to draw general conclusions. For this reason we discuss only a few recent examples.

The herbicide imazaquin had no effect on microbial enzyme activity estimated by FDA hydrolysis and TTC dehydrogenase assays (Seifert et al. 2001). Soil treatment with the herbicide brominal and the insecticide selecron (1) stimulated acid phosphatase activity at field application rates but inhibited this enzyme activity at the higher application doses; (2) generally inhibited arylsulfatase activity; and (3) stimulated alkaline phosphatase activity even at the higher application rates (Omar and Abdel-Sater 2001). A direct role of alkaline soil pH in increasing resistance of alkaline phosphatase to the pesticides was suggested.

The insecticides dichlorovos, phorate and methomyl applied to soil at 2.5 kg ha^{-1} and chloropyrifos and methyl parathion applied at 5.0 kg ha^{-1} increased phosphatase activity up to 20 days of incubation and the enzyme decreased progressively on prolonging the incubation period (Madhuri and Rangaswamy 2002). Metsulfuron-methyl significantly reduced amylase, invertase and xylanase activities for the entire study period (28 days) in loamy sand and clay soils under laboratory conditions (Ismail et al. 2000).

Glyphosate and paraquat increased invertase activity of several soils (Sannino and Gianfreda 2001). These increases ranged from 4–204% and were higher than increases in urease activity. Phosphatase activity was generally inhibited (up to 98%) by glyphosate. The effects of atrazine and carbaryl were markedly affected by methanol, the solvent used for their solubilisation. It was observed that the higher the clay+pH value of soils, the greater the residual activity of phosphatase and the lower the inhibition effects of pesticides.

Other Stresses

Acid phosphatase, β-glucosidase chitobiase and phenol oxidase activities were chosen as bioindicators of the effect of a single, dormant season prescribed fire in a southern Ohio hardwood forest (Boerner et al. 2000). Postfire acid phosphatase activity was generally reduced by burning and decreased with increasing long-term water potential. Postfire β-glucosidase activity differed little between control and burned plots. Chitinase activity increased postfire in proportion to fire intensity. Phenol oxidase activity was highly variable and did not correlate well with either fire or soil water potential. It was suggested that the use of prescribed fire for the restoration of a forested ecosystem consumed the majority of leaf litter and changed the quality and quantity of organic matter of the forest floor without significant changes in microbial community structure or metabolic profiles, at least to the degree that soil enzyme activity mirrored those attributes.

Soil enzyme activities have also been measured to assess the effect of the stress due to the conversion of forest soils into arable soils with a range of vegetation types (Caldwell et al. 1999). Annual harvest or continuous vegetation removal to bare soil for 4 years reduced β-glucosidase and phosphomonoesterase activities in lowland soils of differing fertility in Costa Rica (Caldwell et al. 1999). However, conversion from tropical forest into pineapple plantation with plant biomass incorporation into soil every 3–4 years did not increase most lignocellulose degrading and nutrient releasing enzyme activities (Waldrop et al. 2000).

Simulated acid rain (a mixture of H_2SO_4 and NH_4NO_3) added at different doses to forest tree canopies for 4 years reduced soil phosphatase activity compared to soil under control trees (Carreira et al. 2000). It was suggested that the effects of acid rain induced P deficiency and reduced tree growth.

Microbial biomass, dehydrogenase and arylsulfatase activity were decreased by fluorine contamination and the most sensitive parameter was arylsulfatase activity (Tscherko and Kandeler 1997). The ratio of arylsulfatase to microbial biomass was suggested as a sensitive index for evaluating environmental stress due to fluorine contamination.

12.6 Effects of Land Management Practices on Soil Enzyme Activities

Several parameters, such as C, N, P contents, pH, texture, metabolic quotient, biomass, SIR, ATP content, enzyme activities (Alef and Nannipieri 1995; Leirós et al. 1999; Nannipieri 1994; Trasar-Cepeda et al. 1998) and, recently, molecular and physiological characterisation of microbial community structure and diversity (Amann and Ludwig 2000; Doran and Zeiss 2000; Hill et al. 2000; Morris et al. 2002; Ogram 2000), have been monitored to assess the effect of management practices on soil quality. Enzyme activities, as well as microbial biomass and microbial respiration, are often used as early indicators to monitor the microbial response to soil amendments and to evaluate the sustainability of land management practices.

12.6.1 Nitrogen Fertilisation

Studies on the effect of inorganic N fertiliser applications on enzyme activities have led to contradictory results. For example, Eivazi and Tabatabai (1990) found that addition of NO_3^--N during the assay partially inhibited

β-glucosidase activity. In contrast, in a greenhouse experiment involving corn crops, β-glucosidase activity was proportional to the amount of NH_4NO_3-N added after 164 days (Fauci and Dick 1994), whilst L-histidine NH_3-lyase and protease activities were not affected by inorganic N. Probably, added N stimulated the release of root exudates which in turn stimulated β-glucosidase activity, whereas uptake by plant roots kept the amount of inorganic N in the soil solution low, thus reducing the risk of enzyme inhibition.

Recently, Dodor and Tabatabai (2003) have shown that only one (amidase) of four amidohydrolases was significantly affected by N fertilisation in soils of two long-term cropping systems. Both urease and dipeptidase activities and microbial biomass level decreased in soils receiving long-term N fertilisers compared to native soils or soils treated with organic matter inputs (Burket and Dick 1998), probably due to the acidifying effect of N fertilisers (Dodor and Tabatabai 2003). Acid and alkaline phosphatases, α-glucosidase, arylsulfatase, and urease activities increased when organic and inorganic N fertilisation were added together (Eivazi et al. 2003). Probably, the stimulation of β-glucosaminidase activity by N fertilisation depended on the increase in chitin concentration due to microbial growth stimulated by fertiliser addition (Ekenler and Tabatabai 2002a).

Both extracellular and intracellular arylsulfatase and urease activities were not significantly affected by N fertilisation (Klose et al. 1999; Klose and Tabatabai 2000). Urease activity decreased probably due to the uptake of mineral N by soil microorganisms that made the synthesis of urease unnecessary (Dick et al. 1988b; Burket and Dick 1998; Marcote et al. 2001), thus confirming the hypothesis of Konig et al. (1966) that high amounts of NH_4^+ decrease urease activity.

The lack of a certain trend is not surprising considering that the effects of inorganic fertilisers on soil enzymatic activities in field experiments vary with soil management practices, cropping, time, soil properties, composition and amount of the fertiliser applied and enzyme assayed. In addition, fertilisation can affect soil physical properties with increases in soil porosity and shrinkage but the effects of such modification on soil enzyme activities are not clear (see Sect. 12.7). Organic matter decomposition can be affected by fertilisation but it is not clear the role of the commonly measured soil enzyme activities in this process.

However, as discussed above, the most important drawback is that the present enzyme assays do not distinguish at least intracellular versus extracellular enzyme activity, and thus a correct interpretation of enzyme measurements is not possible.

12.6.2 Organic Amendments

In general, recent additions of organic residues can stimulate enzyme activities as the result of microbial proliferation or enzyme induction in response to the amendment, if inhibitory substances are not present in organic residues such as heavy metals in municipal solid waste (MSW) compost. Indeed the ratios between acid phosphatase, alkaline phosphatase, α-glucosidase, arylsulfatase, or urease activity and microbial biomass were significantly increased by manure regardless of crop type in historic Sanborn field (Missouri) soil under various cropping and management practices since 1888 (Eivazi et al. 2003). However, repeated annual organic fertilisation can usually lead to lower levels of enzyme activity (Perucci 1992; Marcote et al. 2001) even if constant values were found in some long-term experiments because constant and regular organic addition balanced stimulation and suppression of enzyme activity (Martens et al. 1992).

The activities of β-glucosidase, nitrate reductase, dehydrogenase, urease, phosphatase and protease of cultivated plots, treated with 12 tons ha^{-1} of compost, were slightly higher than those of control soil after 6 years. However, a double dose did not induce any further increase, whereas it reduced protease activity probably because it was more sensitive to the heavy metals introduced by compost amendments than the other enzyme activities (Crecchio et al. 2004). It was suggested to avoid large application rates of liquid pig manure to soil under continuous silage corn monoculture because they decreased acid and alkaline phosphatase, arylsulfatase, urease and dehydrogenase activities with a decline in microbial counts (Lalande et al. 2000).

The increased level of enzyme activities in the organic-amended soil may be a reflection of the increased protective sites within the soil as a result of enhanced humus content (Martens et al. 1992; Pascual et al. 1999).

The inhibitory effect of heavy metals can overcome the stimulatory effect of the MSW compost addition to soil (Nannipieri 1994; Marcote et al. 2001) and the effect depends on the different sensitivity of enzymes to heavy metals (Dick 1992; Perucci 1992; García-Gil et al. 2000). Other processes involved in the effects of MSW composts on soil enzyme activity are repression or stimulation of enzyme synthesis (Nannipieri 1994) and complexation of heavy metals by mineral and organic components which affect the amount of bioavailable heavy metal.

In field experiments, the measured effects are also dependent on the cropping system, the experimental design adopted, the sampling times, the agricultural management, the climatic conditions, and the stability of fresh versus mature material organic amendments. Contradictory results have often been recorded when short- and long-term effects are compared

(García-Gil et al. 2000; Crecchio et al. 2001). By assaying dehydrogenase activity as an index of microbial activity in soil (Nannipieri et al. 1990) it was found that the application of fresh material compared to mature compost led to a rapid flush of soil microbial activity in the first stages, followed by a decrease probably due to the decreased availability of more easily degradable substrates (Serra-Wittling et al. 1996; Pascual et al. 1998; Saviozzi et al. 2002).

Debosz et al. (1999) reported that temporal variations of β-glucosidase, endocellulase and cellobiohydrolase activities driven by climatic conditions (e.g. temperature and moisture factors) and crop growth were more important than differences in organic inputs in soil of arable farming systems.

As mentioned above, different enzyme activities can respond in a different way. Indeed, urease activity of the rhizosphere of potato plants under field conditions was increased by yard waste compost amendment whereas no significant effects on invertase activity were observed (Antonius 2003). Sludge transformation through earthworms (vermicomposting) produces a high-quality organic amendment and prevents the accumulation of toxic compounds. Treatments with vermicompost, mineral (N-P) and organo-mineral increased V_{max} values of dehydrogenase activity of soil even 1 year after the treatments, whereas K_m values revealed a reduction of the enzyme substrate affinity only after mineral fertilisation, probably due to a change in the composition and activity of soil microbiota (Masciandaro et al. 2000b).

Cellobiohydrolase, β-*N*-acetylglucosamidase, β-glucosidase, urease and acid phosphatase activities but not invertase and xylanase activities were significantly increased in dairy slurry treatments after 2 weeks (Bol et al. 2003). Increases in enzyme activities were suggested to be due to the improvement in soil conditions (such as reaching a pH value near to optimal one) and due to the application of ureolytic microorganisms by the slurry amendment in the case of urease activity. Probably xylanase activity was not affected because microbial synthesis was not stimulated (Bol et al. 2003).

Quantitative and qualitative differences in substrate supply can lead to significant variations in the composition and activities of soil microbial communities. It is noteworthy to say that techniques such as genetic fingerprinting of soil DNA, signature phospholipid fatty acids, and carbon substrate utilisation (Biolog), confirm that long-term addition of organic amendments may influence the microbial community structure and such shifts are not necessarily accompanied by changes in enzyme activities (Marschner et al. 2003). The functional redundancy of soil microorganisms as well as the long-term adsorption of enzymes on soil particles, and the measurement of the potential activity instead of the actual activity by the laboratory assays, may account for the absence of changes in enzyme activities following changes in microbial community structure.

12.6.3 Tillage, Cropping and Other Managements

Soil management influences microbial activity, microbial biomass and enzyme activity through changes in nutrient inputs and in the quantity and quality of plant residues entering the soil (Burns 1978a; Ekenler and Tabatabai 2002a). Different tillage significantly alters soil properties and especially the spatial distribution within the soil profile of the various enzyme activities (Deng and Tabatabai 1996a; Bergstrom et al. 1998a,b). Usually, enzyme activities of the surface layer of no-till soils are higher than those of the same layer of tilled soils, whereas the opposite occurs for the deepest soil layers (Angers et al. 1993; Kandeler and Böhm 1996; Bandick and Dick 1999; Kandeler et al. 1999a,b; Roscoe et al. 2000; Dumontet et al. 2001) and this depends on the fact that microbial activity of surface no-till soils is significantly greater than in conventional tillage field, whilst the opposite occurs in the deeper soil layers. However, tillage effect depends on enzyme activity since urease activity was less sensitive than phosphatase activity to soil degradation induced by a tillage system and crop rotation (Palma et al. 2000). However, the response of enzyme activity can be annually and seasonally different (Kandeler and Böhm 1996; Curci et al. 1997). Also soil topography can be important and confound tillage effects (Bergstrom et al. 1998a, 2000).

Conventional tillage accelerates the microbial oxidation of organic matter thus stimulating a greater microbial activity. For example, microbial activity, estimated by FDA hydrolysis and dehydrogenase activity, was greater under conventional tillage than by incorporating plant residues into the surface soil, due to more labile C substrates supporting microbial activity (Seifert et al. 2001). However, the effect depended on the considered soil horizon.

Both tillage and management systems can indirectly affect enzyme activities through changes in the structure of microbial communities. Differences in enzyme activities between the fine sandy loam, the sandy clay loam and loam soil, paralleled differences in the fatty acid methyl ester profiles (Acosta-Martinez et al. 2003). Changes in acid and alkaline phosphatases, dehydrogenase, FDA hydrolysis, β-glucosidase, and urease activities induced by tillage of arable soils in Argentina were related to the main physiological groups of bacteria and fungi and to water-filled pore space, organic C and total N contents (Aon and Colaneri 2001; Aon et al. 2001).

Studies on the effects of three tillage systems and four residue-management practices on amidohydrolase, glycosidase, phosphatase, and arylsulfatase activities showed that these enzyme activities were significantly greater in no-till/double mulch than in most of the other tillage and residue-

management practices investigated (Deng and Tabatabai 1996a,b, 1997). Mulching of the soil surface reduced evaporation losses and increased the soil water-holding capacity, thus favouring microbial growth and enzyme production.

The type of crop can also affect soil enzyme activities. Generally, monoculture causes the physical degradation of soil, with possible negative effects on soil microbiological properties, whereas crop rotation positively influences soil microbial activity. The high input and diversity of organic materials generally stimulate microbial activity leading to higher concentrations of microbial biomass and enzyme activities than monocropping systems (Miller and Dick 1995; Friedel et al. 1996; Burket and Dick 1998; Klose et al. 1999; Klose and Tabatabai 2000; Ekenler and Tabatabai 2002a; Dodor and Tabatabai 2003). Other factors affecting enzyme activity are crop type and the extension of root system of crops and the consequent rhizosphere effect. Amidase, arylsulfatase, deaminase, FDA hydrolysis, invertase, cellulase and urease activities were generally higher in continuous grass and pasture than in cultivated fields (Bandick and Dick 1999). The α- and β-glucosidase and α- and β-galactosidase had the highest activity in the soil under cover crops, followed by soil under the fescue and then by soil under winter fallow treatments. In cultivated systems, enzyme activity was higher where organic residues were added as compared to treatments without organic amendments. Enzyme activities were higher in native grassland than in forest soils (Saviozzi et al. 2001).

Soil managed under permanent grass supported approximately 150% greater dehydrogenase activity and 180% greater acid phosphatase activity than no-till and conventionally tilled soils, which showed similar enzyme activities (Carpenter-Boggs et al. 2003). The type of crop is not always the main factor affecting soil enzyme activity. For example, while arylamidase activity was significantly influenced by crop rotation at one site, no relationship was found at the second site probably due to the high spatial variability and the sampling time (Dodor and Tabatabai 2002).

Dodor and Tabatabai (2003) claimed that "the response of different enzyme activities to cropping systems is most likely related to the development of the microbial community structure and influenced by soil properties and environmental factors". As previously reported (see Sect. 12.2), distinct soil microbial communities develop under different plant species (Grierson and Adams 2000). Different plant species, particularly exotic plants, altered rapidly (within 3 months in greenhouse experiments) both the community structure (as indexed by PLFA profiles) and microbial function (as indexed by the responses of SIR and eight enzyme activities) of soil (Kourtev et al. 2003). The plant growth stages also had an important impact on soil enzyme activities and microbial populations (Li et al. 2002).

12.7 Relationship Between Enzyme Activities and Soil Physical Properties and Soil Depth

Soil enzyme activity is generally related to soil microbial population size and it is positively influenced by the amount of pores ranging from 30–200 μm (Pagliai and De Nobili 1993). Marinari et al. (2000) showed that the addition of organic fertilisers improved soil physical and microbiological properties. They reported a significant linear correlation between acid phosphatase and dehydrogenase activities and soil total porosity, whereas benzoylargininamide hydrolysing activity was not correlated with any physical parameter.

Relations between soil enzyme activities and bulk density depended on the enzyme type and plant growth stage (Li et al. 2002). It was concluded that maize crop growth and yield responses to soil bulk density generally followed the same pattern as the activities of most enzymes, implying the significant role of the measured enzyme activities in nutrient transformation in soil. Dick et al. (1988a) reported a significant negative correlation of dehydrogenase and phosphatase activities with soil bulk density of the 10–20 m layer of a forest soil.

Generally, enzyme activities decrease with soil depth (Deng and Tabatabai 1996a,b, 1997; Ekenler and Tabatabai 2003a,b) as it does microbial biomass and organic C content. Protease, urease, and deaminase activities decreased with soil depth of the slurry-amended grassland, but they were significantly higher than those of control at different soil depths due to the stimulation of microbial growth in response to leaching of low molecular weight organic compounds through soil profile (Kandeler et al. 1994). The activities of arylsulfatase, β-glucosidase, phosphomonoesterases, dehydrogenase, and FDA hydrolase, assayed with or without buffers, decreased with depth following the same pattern of bacterial number and organic matter content (Taylor et al. 2002). The exception was non-buffered and buffered urease activity, which was retained and protected by clays. The decrease in enzyme activities with increasing depth was also observed in rhizosphere soil with the exception of acid phosphatase activity, which was similar at all depths, probably because these enzymes are mainly secreted by plant roots (Naseby and Lynch 1997).

12.7.1 Distribution of Enzyme Activities with Soil Particles

Enzyme activities have been measured in soil particles to better understand the relationships between biotic and abiotic constituents with enzyme

activity of soil, by using a preservative fractionation method by Stemmer et al. (1998), which is quite soft to allow complete recovery of soil enzyme activities (Kandeler et al. 1999a,b,d; Gerzabek et al. 2002; Poll et al. 2003). In the case of xylanase the sum of the enzyme activities of all isolated fractions considerably surpassed the activity of the bulk soil likely due to aggregate breakdown releasing new enzymatically active surfaces (Stemmer et al. 1998). The same work conducted by using low-energy sonication showed an enrichment of invertase activity in the silt- or clay-sized fractions in relation to the organic C content. Urease activity was mainly located in the smaller fractions, whilst xylanase activity was enriched in sand fractions. Different rates of organic and inorganic amendments increased urease activity in all particle-size fractions, whilst the increase of xylanase activity was variable depending on the fraction when particle-size fractions were prepared by a long-term managed soil (organic and inorganic fertilisation, crop rotation; Kandeler et al. 1999b). The highest enzyme activity-to-C ratios were observed in the smaller fractions and in the coarse particles.

The silt-sized particles were the principal medium-term sink for organic C, applied as organic manures and/or plant residues to an Eutric Cambisol treated for 42 years (Gerzabek et al. 2002). The sand-sized fractions accumulated organic C by increasing organic C levels of bulk soil, thus possibly acting as indicators of organic C content of the investigated soils, whereas the clay-sized fraction contained more stable organic C and was less affected by treatments than the other particle-size fractions. Invertase activity was found largely in silt- and clay-sized particles, whereas xylanase was found in fine sand particles. An increasing bacterial diversity and abundance with decreasing particle size was also evidenced by 16S rRNA-based analysis (Gerzabek et al. 2002). Both fungal and bacterial communities were differently distributed among coarse sand, silt and clay fractions of a Luvic Phaeozem soil subjected to farmyard manure application over 120 years (Poll et al. 2003).

Xylanase activity was mainly located in the coarse sand fraction of the conventional tillage treatment and alkaline phosphatase activity in the silt and clay fraction independently of tillage treatment (Kandeler et al. 1999d). Protease activity was higher in the coarse and in the clay fractions and invertase activity in the silt fraction of the reduced and minimum tillage treatments. Cultivation reduced the activities of arylsulfatase and acid phosphatase in all aggregate size fractions (Gupta and Germida 1988). Microaggregates of both native and cultivated soils contained lower enzyme activities than their respective macroaggregates.

The highest numbers of microorganisms and the highest β-glucosidase and β-acetylglucosaminidase activities were found in organic particles of a long-term (100 years) wastewater irrigated soil (Filip et al. 2000), whereas protease activity was concentrated in the smaller organic and silt+clay

fractions despite the decrease in microbial counts in these two fractions. The overall effects remained detectable even 20 years after the wastewater irrigation was terminated.

In soil polluted by heavy metals, urease activity was mainly located in the clay fraction, alkaline and arylsulfatase activities in the silt-sized and clay particles, and xylanase activity was almost equally distributed among the particle-size fractions, with a slightly lower activity in the clay fractions. In agreement with earlier studies, the predominance of enzyme activities in different size fractions appeared independent of soil type, soil management and soil pollution (Kandeler et al. 2000).

Both invertase and xylanase activities of silt and clay fractions were hardly affected by plant litter amendment, whereas the enzyme activity of the coarser fractions containing most of the particulate organic matter was directly affected by litter quantity and quality (Stemmer et al. 1999). These studies demonstrated that several soil microhabitats, other than rhizosphere (Sect. 12.2), have fairly distinct physical, chemical and microbiological properties that operate at different spatial scales and support the temporal and spatial heterogeneity of microbial communities (Beare et al. 1995). Fresh organic residues added to soil offer new microbial sites. Thus at the litter–soil interface (detritusphere) a stimulation of the microbial growth was accompanied by an increase in protease, invertase and xylanase activities which exponentially decreased at a distance of a few millimetres from the litter surface probably corresponding to the depletion of dissolved organic C (Kandeler et al. 1999c).

12.8 Effects of Transgenic Plants and Recombinant Microorganisms on Soil Enzyme Activities. The Potential Role of Rhizosphere Enzyme Activities

There are several concerns about the possibility of horizontal gene transfer between genetically modified plants and the soil microbial community. The hypothetical acquisition of these genes by the bacterial community raises great concern about the undesirable effects of altering the resistance level of indigenous bacteria and of disseminating such traits into the environment. For example, *Acinetobacter* spp., the phytopathogen *Ralstonia solanacearum*, and the bacterial pathogen *Erwinia chysanthemi* can capture at very low frequencies and in soil free microcosms, fragments of DNA from plant homogenates, purified genomes or cloned parts of transgenic plants such as tomato, potato, oilseed rape, sugar beet, and tobacco (Schlüter et al. 1995; Nielsen et al. 1997, 2000, 2001; Gebhard and Smalla

1998, 1999; Bertolla et al. 2000; De Vries et al. 2001; Nielsen and van Elsas 2001; Kay et al. 2002). Although experiments have been based on the use of pure bacterial cultures often in sterile soil, it is reasonable to suppose that transformation-mediated gene transfers in soil can involve a higher number of bacteria than previously thought.

Enzyme activities have been measured to study the effect of transgenic plants on soil metabolism. Both dehydrogenase and alkaline phosphatase activities of soil sampled from transgenic alfalfa, regardless of association with recombinant N-fixing soil *Sinorhizobium meliloti*, were significantly lower than those of soil sampled from parental alfalfa (Donegan et al. 1999). Wei-xiang et al. (2004) found that, under laboratory conditions, dehydrogenase activity was increased by the incorporation of Bt-transgenic rice straw in a flooded paddy soil with respect to soil treated with non-Bt straw because the addition of the transgenic plant increased the availability of readily metabolisable plant substrates. In contrast, no differences between the activities of soil neutral phosphatase were recorded between non-Bt straw- and Bt-straw-amended soils.

Determination of rhizosphere enzyme activities has been proposed as an alternative way of monitoring perturbation resulting from the introduction of genetically modified microorganisms in the ecosystem (Naseby and Lynch 1998). The inoculation of wheat seeds with a genetically modified strain of *Pseudomonas fluorescens* increased urease and chitobiosidase activities of rhizosphere soil at 0–20 cm depth and decreased alkaline phosphatase but not acid phosphatase activity (Naseby and Lynch 1997). The reduction in alkaline phosphatase activity was attributed to a displacement of the rhizosphere communities producing the enzyme. Opposite changes in the measured enzyme activities were observed when inoculation of wheat seeds with *P. fluorescens* was carried out in the presence of a mixture of urea, chitin and glycerophosphate (Naseby and Lynch 1997).

The *P. fluorescens* strain F113, which is a wild-type producing the antifungal 2,4-diacetylphloroglucinol (DAPG) and which was marked with a *lacZY* gene cassette, increased alkaline phosphatase, phosphodiesterase and arylsulfatase activities of pea rhizosphere whereas the other inocula reduced enzyme activities compared to the control (without bacterial inoculum; Naseby and Lynch 1998). It was suggested that increases in enzyme activities were caused by the production of DAPG, which decreased the available inorganic phosphate and sulfate in the rhizosphere being the synthesis of these enzymes controlled by these nutrients (Tabatabai 1994; Naseby et al. 1998). However, an opposite trend was found for acid phosphatase activity, which, in contrast to alkaline phosphatase (microbial origin) activity, is mostly of plant origin and, therefore, more dependent on the nutritional status of the plant. The presence of the F113 strain was associated to the lowest β-galactosidase, β-glucosidase, N-acetylglucosaminidase activities

and probably this behaviour depended on the increase in available C. On the other hand, no effects on enzyme activities were observed when *P. fluorescens* F113 was present in the rhizosphere of field-grown sugar beet (Naseby et al. 1998). It was concluded that the impact of various genetically modified *Pseudomonas* spp. on the rhizosphere populations and functions depended on the nature of the genetic modification (Naseby and Lynch 1998).

The potentialities of enzymes produced by rhizosphere microorganisms, including genetically modified microorganisms, in bioremediation and biocontrol of pests and diseases have been discussed by Naseby and Lynch (2002).

12.9 Relationship Between Enzyme Activities and Their Substrates or Products in Soil

Inducible enzymes are enzymes whose synthesis is induced by the presence of substrates. For example, cellulase activity in soil was found to be correlated with the cellulose content (Pavel et al. 2004) and N-related enzyme activities were related to low N availability in soil (Kourtev et al. 2003). Xylanase and invertase activities in decomposing green tissue in soil depended on the amount of substrate, indicating a possible relationship between the two enzymes (Luxhøi et al. 2002). The amendment of soil cores with a mixture of urea, chitin and glycerophospholipid increased the activities of urease, chetobiosidase and N-acetylglucosaminidase, respectively (Naseby and Lynch 1997). The absence of effects of glycerophosphate addition on phosphatase activities was probably due to the rapid hydrolysis to phosphate, which represses the synthesis of the enzyme (Nannipieri et al. 1990; Acosta-Martínez and Tabatabai 2000; Ekenler and Tabatabai 2003b). Sometimes enzyme repression occurs by compounds produced from reaction products as in the case of microbial production of urease in C-amended soils (McCarty et al. 1992).

Polyacrylamide (PAM) is currently used as an irrigation water additive to significantly reduce soil erosion and can be used as an N source by soil microorganisms through the intracellular and extracellular PAM-specific amidases (Kay-Shoemake et al. 1998, 2000). These enzymes are inducible and not constitutive since they are not produced in cultures supplied with only ammonium nitrate.

12.10 Enzymes as Decontaminating Agents

Transformation reactions of phenols and chlorophenols by laccases and peroxidases have widely been investigated under laboratory conditions. Their oxidative self-coupling (Dec and Bollag 2000; Durán and Esposito 2000; Bollag et al. 2003; Gianfreda et al. 2003) and cross-coupling reactions with humic precursors (Filazzola et al. 1999; Park et al. 1999; Itoh et al. 2000), humic substances themselves (Huang et al. 2002; Park et al. 2000), and soil-derived dissolved organic matter (Huang and Weber 2004) demonstrated the effectiveness of laccases and peroxidases in reducing the mobility of phenolic contaminants in soils. Experiments carried out using C-14-labelled phenols demonstrated that the reduction in mobility was brought about by the polymerisation of phenols and subsequent adsorption of the polymers on soil particles (Bhandari and Xu 2001). The two enzymes were also effective in the transformation of the herbicide bentazon, a relatively inert chemical, in the presence of humic monomers as representative electron donor co-substrates in oxidative coupling reactions (Kim et al. 1998).

Gramss (1997) and Gramss et al. (1999b) reported that basidiomycetous mycelia grown in grassland and forest soils exhibited several cell-free polyphenol oxidases, peroxidases, manganese peroxidases, hydroxylases, aromatic-ring cleavage dioxygenases, and esterases as well as active oxygen species and, in part, lignolytic peroxidases. The activities of these enzymes catalysed the degradation of lignin, humic material, and aromatic xenobiotics by hydroxylation, dechlorination, oxidation, and cleavage of aromatic ring structures (Gramss 1997).

The contamination of a semiarid Mediterranean soil by hydrocarbons from oil sludges increased dehydrogenase, urease, protease and phosphatase and microbial activities and improved soil structure (Caravaca and Roldán 2003). This indicated a pronounced ability of the soil microbial communities to degrade organic substrates in the contaminated soil.

Very low levels of enzyme activities and a different bacterial diversity as assessed by DGGE profiles of the 16S rDNA were found in soils heavily polluted by polyaromatic hydrocarbons (PAHs) as compared to an uncontaminated soil (Andreoni et al. 2004). By comparing the biodegradation of PAHs in unsterile and sterile soils, as well as in uninoculated and inoculated soils, the importance of fungi, bacteria, soil organic matter and lignolytic enzymes in the degradation process was shown (Gramss et al. 1999b).

Different actinomycete species of soil degraded the herbicide Diuron up to 37% in 7 days through manganese peroxidase activity (Esposito et al. 1998). Triazine herbicides were microbially metabolised by hydrolases.

Atrazine chlorohydrolases initiated the metabolism of the herbicide by catalysing a hydrolytic dechlorination reaction to produce hydroxyatrazine. Enzymes from the amidohydrolase family catalysed the hydrolytic removal of substituents from the s-triazine ring and produced cianuric acid as an intermediate, a compound metabolised by a variety of soil bacteria via a series of amydases able to yield C and N as carbon dioxide and ammonia, respectively (Seffernick et al. 2002; Wackett et al. 2002). Recombinant *E. coli* strains expressing atrazine chlorohydrolase have been constructed and biotechnologically exploited for atrazine remediation in soil (Wackett et al. 2002; Wackett 2004).

12.11 Enzyme Activities as Indicators of the Functional Status of the Soil Community

Often single enzyme activities have been measured to assess soil fertility or soil quality. For example, dehydrogenase and β-glucosidase activities were sensitive indices of changes in soil organic matter content, viable microbial counts (Nannipieri 1994; Masciandaro et al. 2000a; Wei-xiang et al. 2004), and vegetation cover (Caldwell et al. 1999). Acid and alkaline phosphatases and microbial biomass C seemed to be the best indicators of the effects of long-term cropping and management practices on soil quality (Jordan et al. 1995). Xylanase activity was identified as a very sensitive indicator of tillage practices (Vaughan et al. 1994; Kandeler et al. 1999b); β-glucosaminidase activity, involved in chitin degradation in soils, was used as an index of net N mineralisation in soils and was significantly affected by soil disturbance, tillage and residue management practices (Ekenler and Tabatabai 2002a, 2003c). Among amylase, chitinase, xylanase, and β-glucosidase activities, β-glucosidase activity was the most sensitive indicator for showing differences between fallows in the semiarid tropical regions, whereas no clear relationships between soil enzyme activities and soil organic matter and total microbial biomass contents were found (Badiane et al. 2001). Considering the seasonal variability of the parameters at two sites with contrasting histories of land-use management and degree of degradation, Wick et al. (2002) concluded that: (1) alkaline phosphomonoesterase activity was steady over time and, thus, considered a suitable indicator with which to monitor long-term changes of soil quality; (2) microbial biomass and β-glucosidase activity fluctuated markedly, but seasonal and temporal variations at degraded and non-degraded sites were similar and, thus, they were considered sensitive indicators to monitor short-term variations of soil quality; (3) acid phosphomonoesterase and protease activities fluctuated during the course of a year and exhibited pronounced interseasonal

differences; thus, neither were considered a sensitive indicator of changes in soil quality over time.

However, it is conceptually wrong to assume that a single enzyme activity should be indicative of the overall state of nutrients and microbial dynamics in soil because, e.g., microbial activity includes a very broad range of enzyme activities (Nannipieri 1994). Thus it has been suggested that measurements of more microbial and biochemical parameters should be made and such values should be integrated in a single index. According to Masciandaro et al. (2000a) the ratio between dehydrogenase activity and water-soluble C better represented metabolic activity of soil. An alkaline phosphatase/acid phosphatase (AlkP/AcdP) ratio value of approximately 0.5 could separate soils with adjusted pH value due to lime application from soils still requiring additional pH adjustment (Dick et al. 2000).

Various indices of soil biochemical quality, composed of a minimum data set, have been proposed from a combination of biological and biochemical parameters closely correlated with organic C and total N contents. According to Trasar-Cepeda et al. (1998) the selection of the most appropriate variables should consider the following criteria: (1) the multiple correlation coefficient of the equation should be as close as possible to unity; (2) the number of variables in the final equation should be as low as possible; and (3) the parameters should be measurable by simple, accurate and rapid methods.

A number of empirical relationships, often based on the multiple regression model and combining different soil properties, have been proposed as useful indices of soil biochemical quality (Table 12.4). It is noteworthy that in all the four proposed indices the activity of β-glucosidase was considered. According to Trasar-Cepeda et al. (2000) the index resulting from the combination of phosphomonoesterase, β-glucosidase and urease activities, microbial biomass C, N mineralisation capacity, and total N content was sensitive to pollution by tanning effluent, hydrocarbons or landfill effluent.

Moreno et al. (2001) described mathematical models to calculate the ecological dose ED (expressed as ED_5, ED_{10} and ED_{50}) values of Cd and Ni for urease and phosphatase activities. It was suggested that the ED values be used to quantitatively assess the impact of heavy metals on soil functioning.

Canonical discriminant analysis (CDA) of seven enzyme activities of control soils and soils affected by anthropogenic activities (Rao et al. 2003) showed that enzyme activities were able to discriminate between altered and unaltered soils irrespective of the site. Numerical alteration indices were developed as a linear function of the seven enzyme activities.

Table 12.4. Empirical mathematical relationships including enzyme activities

Dependent parameter	Independent parameters	Mathematical expression	Reference
Organic matter content	Microbial biomass C and β-glucosidase and arylsulfatase activities	Organic matter (g kg^{-1}) = 5.35 + 0.017 (microbial biomass C, μg g^{-1}) + 0.06 (β-glucosidase, μmol p-nitrophenol g^{-1} h^{-1}) + 1.08 (arylsulfatase activity, μmol p-nitrophenol g^{-1} h^{-1})	García and Hernández (1997)
Total N content	Microbial biomass C and mineralised N and phosphomonoesterase, β-glucosidase, and urease activities	Total N (% w/w) = 0.38×10^{-3} (microbial biomass C, mg kg^{-1}) + 1.4×10^{-3} (mineralised N, mg kg^{-1}) + 13.6×10^{-3} (phosphomonosterase, μmol of liberated product g^{-1} h^{-1}) + 8.9×10^{-3} (β-glucosidase, μmol of liberated product g^{-1} h^{-1}) + 1.6×10^{-3} (urease, μmol of liberated product g^{-1} h^{-1})	Trasar-Cepeda et al. (1998)
Inorganic N taken up by ryegrass	Total N and β-glucosidase activity	Mineralised N uptake by ryegrass = –12.38 + 0.23 (β-glucosidase, mg of liberated product kg^{-1} h^{-1}) + 0.023 (total N, mg kg^{-1})	Burket and Dick (1998)
Organic C content	Arylsulfatase, dehydrogenase, phosphomonoesterase and β-glucosidase activities	Organic C (g kg^{-1}) = –0.4008 (arylsulfatase, mg p-nitrophenol kg^{-1} h^{-1}) + 0.4153 (dehydrogenase, mg INTF kg^{-1} 24 h^{-1}) + 0.4033 (acid phosphomonoesterase, mg p-nitrophenol kg^{-1} h^{-1}) + 0.4916 (β-glucosidase, mg p-nitrophenol kg^{-1} h^{-1})	de la Paz Jimenez et al. (2002)

12.12 Future Challenges

This review has documented that the interest in enzyme measurements is currently still high. However, it is problematic to relate measured enzyme activities with the real soil functioning. Indeed, as has been highlighted in this chapter, the methodologies adopted to assay soil enzyme activities, under laboratory conditions, still have the following limits: (1) they do not discriminate between the various components contributing to the overall enzymatic activity of soil, and particularly between stabilised and intracellular enzyme activities, the most important ones contributing to the measured enzyme activity; (2) they do not discriminate between enzyme and enzyme-like activities; (3) they measure potential rather than real enzyme activities; (4) they lack standardisation; and (5) they are not able to detect the origin of enzymes in soil. Consequently, it is particularly difficult to explain a change of soil enzymatic activity in response to a certain factor, or to establish the cause-effect relationships between the applied factor and the soil enzyme activity variation. Thus, future research should develop better assays of enzyme activity in soil.

Enzyme activities are also measured to assess changes in soil quality. However, it is conceptually wrong to assume that a specific enzyme activity could represent the multitude of metabolic reactions at the base of soil quality. It is rewarding to combine enzyme activity measurements with other biochemical and microbiological ones. Thus various indices of soil biochemical quality, composed of a minimum data set, have been proposed.

Future research should also address other topics such as: to determine whether enzymes are actively being expressed and whether they are actively catalysing the appropriate reactions without inhibition, once they are expressed. Methods to determine genetic potential, gene expression, as well as direct enzyme activity, adapted to soil conditions are needed. This area of research involves studying the transfer of genetic material which codes for a specific enzyme and the soil factors that affect the expression of the specific enzyme. In this context an advancement has been made by characterising the origin of chitinases by molecular techniques, as discussed in Chap. 11.

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13 How to Assess the Abundance and Diversity of Mobile Genetic Elements in Soil Bacterial Communities?

Kornelia Smalla, Holger Heuer

13.1 Introduction

Genome sequencing data recently confirmed the notion that the horizontal acquisition of genetic modules is a major factor in bacterial evolution and adaptability (De la Cruz and Davies 2000; Ochman et al. 2000; Toussaint and Merlin 2002). Horizontal gene transfer (HGT) is mediated by an uptake of naked DNA (natural transformation; see Chap. 15), by the direct transfer of DNA between cells (conjugation; see Chap. 14), or by integration of DNA transmitted by phages (transduction). Mobile genetic elements (MGE) such as plasmids, bacteriophages, integrative conjugative elements, transposons, IS elements, integrons, gene cassettes and genomic islands play an important role as vehicles for horizontal gene transfer. Table 13.1 summarises briefly the characteristics of the different kinds of MGE and refers to recent reviews. Mobile genetic elements cause micro-diverse patchy populations in soil, which have a common core genome but differ in their flexible genome. Due to its spatial structure and heterogeneity soil stabilises this micro-diversity. Consequently, MGE can help subpopulations to adapt to coevolving biota and environmental stresses, and eventually to colonise empty niches. The exceptional microbial diversity in soils enhances biotic interactions, like competition or facilitation, that may promote evolutionary diversification (Day and Young 2004), and hence the significance of MGE. From a human perspective, it is of importance that MGE played a primary role in the dissemination of antibiotic resistance genes which allowed bacterial populations to rapidly adapt to a strong selective pressure by agronomically and medically applied antibiotics (Tschäpe 1994; Mazel and Davies 1999; Alonso et al. 2001). Horizontal gene transfer was also shown to be important in the evolution of pathogens, as pathogenicity, antibiotic resistance and fitness traits are frequently acquired by HGT (Hacker et al. 1997, 2003). Furthermore, several studies provided evidence that MGE contributed genetic modules to catabolic operons which enable

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Table 13.1. Properties of mobile genetic elements (MGE)

MGE	Properties	Review
Plasmids	Circular or linear extrachromosomal replicons; self-transferable or mobilisable plasmids are vehicles for the transmission of genetic information between a broad or narrow range of species	Thomas 2001
Bacteriophages	Viruses that infect prokaryotes; they can integrate into the host genome and then be vehicles for horizontal gene transfer	Canchaya et al. 2003
Integrative conjugative elements (ICE)	Self-transferable conjugative elements that integrate into the genome of new hosts like temperate bacteriophages; they may promote the mobilisation of genomic islands by utilising conserved integration sites	Burrus and Waldor 2004
Genomic islands	Large chromosomal regions acquired by horizontal transfer that are flanked by repeat structures and contain genes for chromosomal integration and excision	Van der Meer and Sentchilo (2003); Dobrindt et al. 2004
Transposable elements	Genetic elements that can move within or between replicons by action of their transposase; flanked by inverted repeats; transposons typically carry genes for antibiotic resistance or other phenotypes, while IS elements code only for the transposase; multiple copies of the same IS element promote genome plasticity by homologous recombination	Mahillon and Chandler 1998
Integrons	Genetic elements that capture promoterless gene cassettes into an attachment site downstream of a promoter by action of the integrase encoded on the integron; frequently associated with transposons and conjugative plasmids	Hall and Collis 1995

bacteria to degrade recalcitrant manmade compounds in soil (reviewed by Top and Springael 2003).

Mobile genetic elements are undoubtedly important and essential vehicles for bacterial genome plasticity. However, the triggers affecting HGT are largely unknown (Van Elsas et al. 2000; Van Elsas and Bailey 2002; see the Chaps. 14 and 15). Only recently, it was shown by Beaber et al. (2004) that SOS response induced by a variety of environmental factors and antibiotics could increase the expression of genes involved in gene transfer.

The DNA transfer to a recipient's cell will only be successful if the DNA is stably maintained either by integration into the host's chromosome (integration mediated by bacteriophage integrase or an MGE-encoded transposase, or via homologous or illegitimate recombination) or as an autonomously replicating plasmid.

The overwhelming amount of research on the evolution and spread of MGE was clearly done from a medical perspective. However, recent surveys confirmed what could have been expected that, e.g., antibiotic resistance genes localised on MGE are not confined to the clinical environment (Witte 1998; Heuer et al. 2002; Van Overbeek et al. 2002). Indeed bacteria live in every environment and can be moved between different environments (see Fig. 13.1). Soil is thought to be a major sink receiving bacteria and all kinds of pollutants from various sources and the concentration of these pollutants can spatially and temporally change within the soil matrix. Models as to how bacterial populations which temporarily exist in spatial isolation adapt to these stresses are largely lacking. However, once again, MGE also seem to play an important role in the adaptation of soil bacteria in order to allow colonisation of new niches. Our present picture of the diversity and abundance of MGE in soil bacteria is rather incomplete and this is partly due to the fact that only a minor proportion of bacteria are accessible to cultivation techniques (Staley and Konopka 1985; Amann et al. 1995; Smalla 2004).

In this chapter we critically evaluate the potentials and limitations of various different methods, the traditionally used and the more recently

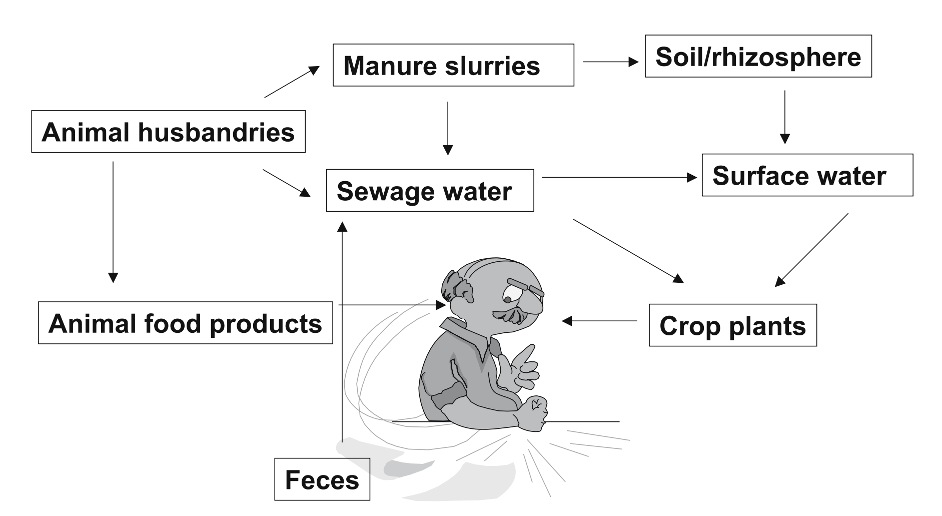


Fig. 13.1. Pathways of mobile genetic elements through diverse environments, horizontally transferring to locally adapted bacterial hosts

developed ones, applied to study MGE in soil bacteria (for review, see Smalla and Sobecky 2002). These methods have been used also to study the MGE in other habitats; however, the focus here will be on their application to soil and some of the challenges which are encountered when working with soil samples will be discussed.

13.2 Cultivation-Dependent Techniques: MGE in Bacterial Isolates from Soil

Traditionally, cultivation-dependent methods were applied to determine the presence of MGE in soil bacteria. Pure cultures of soil bacteria can be isolated either by plating serial dilutions of cell suspensions from bulk or rhizosphere soil samples on non-selective media, or on media supplemented with antibiotics, heavy metals, or xenobiotics. The proportion of soil bacteria accessible to cultivation largely depends on the conditions used. Based on the difference between microscopic counts and plate counts it was estimated that less than 99% of the soil bacteria seem to be non-culturable (Amann et al. 1995). Thus the obvious bottleneck of the cultivation-dependent analysis of MGE in soil bacteria is that the majority of soil bacteria do not form colonies on solid nutrient media. This is supposed to be partly due to the fact that many numerically abundant bacteria are slow growers and often grow only on low-nutrient media. In addition, different kinds of environmental stresses such as UV, drought, high or low temperature might change the physiology of bacteria which are usually culturable, turning them into the viable-but-non-culturable state in which they do not form colonies on plates. New approaches and tricks to get environmental bacteria into culture were recently reviewed by Fry (2004). Although cultivation-dependent analysis of soil bacteria allows only the study of MGE in the proportion of bacteria which form colonies on plates, the obvious advantage is that the bacterial hosts can be identified and thus predictions might be possible with respect to ecological niches. The development and application of molecular tools such as DNA hybridisation, polymerase chain reaction (PCR), and sequencing greatly facilitated studies on the abundance, diversity and evolution of MGE in bacteria.

13.2.1 Plasmids Detected in Soil Bacteria by Plasmid Isolation

To assess the presence of plasmids their DNA can be extracted from the isolates by means of a variety of plasmid DNA extraction protocols (re-

viewed in Smalla et al. 2000c). The different plasmid extraction methods seem to be biased, e.g., with respect to plasmid size, and thus possibly more than one method should be applied to determine the presence of plasmids. Although only a few systematic surveys on the presence of plasmids in soil bacteria have been performed, the general observation is that a considerable proportion of soil bacteria contain plasmids. To determine the phenotypic traits encoded by the plasmid under investigation the plasmid must be either transferred to a well-characterised host or cured from its host. Both strategies can be complicated. If the selectable markers encoded by a plasmid are known, incompatibility testing with a set of reference plasmids can be done. Plasmid DNA can further be analysed by DNA hybridisation (dot or Southern blots, or gene arrays) or PCR. Furthermore, the complete sequence of the plasmid can be determined.

13.2.2 MGE Detected in Soil Bacteria by Probing or PCR

The most feasible and frequently used approaches to screen large numbers of soil isolates for the presence of MGE are DNA-DNA hybridisation studies or PCR amplification with primers targeting MGE-specific sequences (Couturier et al. 1988; Götz et al. 1996; Turner et al. 1996; Smalla et al. 2000b,c; Nield et al. 2001; Stokes et al. 2001). This strategy has been used in different studies to screen bacterial isolates from soil for the presence of broad-host range plasmids, transposons, integrons or phages and provides not only information on the presence of MGE, but also on the kind of plasmid, phage or integron.

Dot- or Southern-blotted genomic or plasmid DNA from soil isolates can be hybridised with labelled probes derived from cloned or PCR-amplified fragments of genes involved in the transfer or replication or integron integrases. Alternatively, total plasmid or phage DNA can be labelled and used as the probe (Lilley et al. 1994; Ashelford et al. 2003).

Colony hybridisation of soil isolates from two sites in the UK with labelled phage cocktails (CP6, PU21) showed that a considerable proportion of the soil bacteria analysed harboured DNA homologous to the phage genomes in the cocktail (Ashelford et al. 2003). Although the presence of phage sequences in soil bacteria does not prove the presence of lytic or temperate phages the authors suggest that bacteriophages in terrestrial ecosystems might play an important role in controlling and stimulating bacterial growth and mediating gene transfer in soil (Ashelford et al. 2003).

High-throughput screening could be achieved by hybridisation of fluorescently labelled genomic or plasmid DNA from isolates with a gene array containing probes (50–70mer or PCR products) for all kinds of MGE (see

also Chap. 9). An array consisting of PCR-amplified open reading frames (ORFs) of the IncP-1α plasmid RK2 and some of the IncP-1β R751 was recently used to determine the similarity of a newly isolated IncP-1β plasmid with the sequenced plasmids (Smalla et al., unpublished).

PCR amplification with primers targeting gene fragments involved in replication or transfer have been successfully used to determine the types of plasmids, or, if done in combination with RFLP (restriction fragment length polymorphism) or sequencing, to estimate plasmid diversity (Götz et al. 1996; Turner et al. 1996; Krasowiak et al. 2002). For some taxonomic groups such as rhizobia, plasmid typing has frequently been used as a tool in diversity studies (Turner et al. 1996). Holmes et al. (2003) examined a collection of *Pseudomonas stutzeri* isolates from soil for the presence of integrons by PCR amplification. The study revealed that integrons were present in some *P. stutzeri* strains but not in all, and that members of *P. stutzeri* exhibit a high degree of gene cassette diversity. The integrons present in *P. stutzeri* chromosomes were shown to possess recombination activity and were suggested to represent hot spots for genomic diversity (Holmes et al. 2003).

13.2.3
Sequencing of MGE in Bacterial Isolates Allows Insight into MGE-Encoded Traits and Their Evolution

With the genomic era, sequencing of complete plasmids became a feasible approach for plasmid characterisation. Before a shot gun clone library is generated, plasmid DNA is usually sheared to sizes of about 1.5–2 kb. The determination of the complete or partial sequences of plasmids provides information required for primer and probe development, for gene array studies, or for site-directed integration of reporter genes. The complete sequence of various plasmids originating from soil isolates has been determined, including the sequence of several so-called megaplasmids from *Sinorhizobium meliloti*, *Ralstonia solanacearum*, and *Agrobacterium tumefaciens*. The latter plasmids often have the size of small bacterial genomes and code for functions involved in the bacteria–host interaction. Recently, the complete nucleotide sequence and organisation was determined for a number of different catabolic plasmids which were isolated from soil bacteria. These catabolic plasmids often carry complete or partial degradative pathways, e.g. for the degradation of 2,4-dichlorophenoxyacetic acid (pJP4: Trefault et al. 2004; pEST4011: Vedler et al. 2004), atrazine (pADP-1: Martinez et al. 2001) or *p*-toluenesulfonate (pTSA: Tralau et al. 2001). While the backbone of those plasmids belonging to the IncP-1 plasmids is remarkably conserved and does not differ between IncP-1 plasmids from clinical

environments (Pansegrau et al. 1994; Thorsted et al. 1998), the phenotypes encoded by the heterologous DNA can be extremely diverse. The catabolic genes were most likely captured via chromosomally located transposons. Plasmids belonging to the IncP-1 group attract a lot of attention because they transfer and stably replicate in a wide range of Gram-negative bacteria even in the absence of selective pressure (Pukall et al. 1996). Therefore, IncP-1 plasmids represent ideal vehicles to assemble genes of interest in various hosts.

13.3 Genome Sequencing of Soil Bacterial Isolates

The sequencing of complete bacterial genomes, as well as comparative genome analysis by macrorestriction mapping, genomic subtraction or microarrays, have clearly shown that a significant proportion of bacterial genes have been acquired by HGT. This evidence has been derived from abnormal sequence similarity (i.e. high similarity of genes from distantly related species), atypical nucleotide composition (guanine+cytosine content, codon usage), incongruent phylogenetic trees, and variation of gene content between closely related strains (Koonin et al. 2001). In many species a high proportion of horizontally transferred genes can be attributed to plasmid, phage or transposon-related sequences since remnants of MGE are often found adjacent to genes identified as horizontally transferred (Ochman et al. 2000).

Data sets of fully sequenced genomes of various soil bacteria have revealed an impressive proportion and diversity of MGE such as IS elements, transposons, integrons, pathogenicity or metabolic islands, integrated phage or plasmid remnants. Forty of 56 sequenced bacterial genomes recently reviewed by Canchaya et al. (2003) contain prophage sequences exceeding 10 kb in length. Bacteria that experienced reductive evolution such as intracellular parasites or symbionts lacked prophage sequences. On small bacterial genomes there obviously is no place for horizontally acquired genes. The bacterial genomes of the two sequenced *Xylella fastidiosa* genomes (Simpson et al. 2000) carry five or six prophages, and prophage occurrence seemed to influence genome evolution. The complete sequence of the soil bacterium *P. putida* KT2440 revealed four different prophages while two prophages were found in the genomes of *P. aeruginosa* and *Xanthomonas campestris*. Another trend noticed by Canchaya et al. (2003) is a lower content of prophages in high G+C Gram-positive bacteria. Not only are numerous virulence factors of bacterial pathogens phage-encoded, but they also have adaptive traits to certain niches.

The complete genome sequence of *Agrobacterium tumefaciens* C58 revealed a total of 24 IS elements and 12 phage-related sequences localised on the circular chromosome (2.84 Mb), the linear chromosome (2.075 Mb) and its two megaplasmids pAtC58 (0.54 Mb) and pTiC58 (0.214 Mb; Wood et al. 2001). This mosaic structure is also evident in genomes of other soil bacteria sequenced or currently being sequenced (*Sinorhizobium meliloti*, *P. syringae*, *P. fluorescens*, *P. putida*, *Ralstonia solanacearum*, *R. metallidurans*, *Burkholderia fungorum*, and *Novosphingobium aromaticivorans*), reflecting a strong selective pressure of soils for genetic plasticity (Galibert et al. 2001; Salanoubat et al. 2002; Buell et al. 2003; dos Santos et al. 2004; http://www.ncbi.nlm.nih.gov/genomes/).

Lan and Reeves (2000) proposed the concept of a bacterial species genome defined by a core set of genes which is shared by a large majority of isolates of a species, and an auxiliary/foreign set of genes. Correspondingly, the latter was also called the flexible genome (Hacker and Carniel 2001), as it is determined by its high plasticity, i.e. gene acquisition and loss. Genes of the flexible genome can confer important ecological traits such as antibiotic or heavy metal resistance, the capacity to degrade xenobiotics, and pathogenic or symbiotic interactions. Genes coding for these traits are often located on MGE or remnants of these.

13.4 Cultivation-Independent Methods

The requirement to develop methods which offered the possibility to study MGE independent from the culturability of their original host resulted from the observation of the so-called plate count anomaly made by Staley and Konopka (1985). Here we would like to give a brief introduction to the methodology and its limitations, and review observations made when these methods were applied to study MGE in soil.

13.4.1 Microscopic Detection of Phages

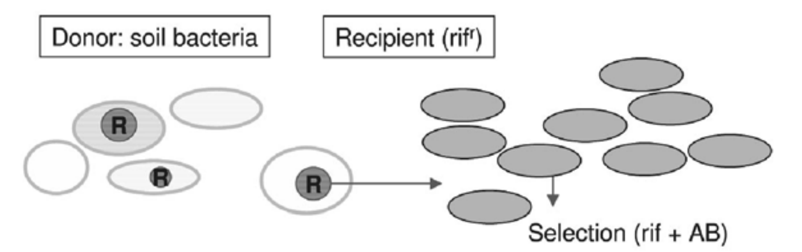
Bacteriophage counts made by means of electron or epifluorescent microscopy for marine or freshwater samples showed that bacteriophage counts were about tenfold higher than bacterial counts. The determination of direct bacteriophage counts in soil is obviously more problematic, and only recently the first report on determination of direct bacteriophage counts in soil showed also a high abundance of viruses in soil (Ashelford et al. 2003).

Methodological development by Ashelford et al. (2003) was needed before reliable counts of bacteriophage in soil could be obtained. Before transmission electron microscopy (TEM) counting, the homogenised rhizosphere and bulk soil suspensions were centrifuged at low speed and filtered through a 0.22-μm membrane. Aliquots of 5 μl were vacuum-dried on electron microscopy copper grid and negatively stained with methylamine tungstate before counting tailed phage and virus-like particles by TEM. Although the direct bacterial counts were similar for both soils the total counts of viruses in soil were significantly higher in Cardiff soil than in Oxford soil. Considering the abrasive nature of soil, Ashelford et al. (2003) suggest that the average count of viruses in soil which was 1.5×10^{7} g^{-1} is probably an underestimate.

13.4.2 Capturing of MGE by Exogenous Isolation in Biparental and Triparental Matings

The idea that is behind the exogenous isolation approach is rather simple, and schemes for the so-called biparental and triparental matings are given in Fig. 13.2. Both types of mating enable the capture of MGE independent from the cultivation of its original host. In a biparental mating (BPM) a selectable recipient strain (rifampicin resistant) is mixed with bacterial cells recovered from an environmental sample such as soil, and incubated on filters (Fig. 13.2a). After the mating the cell mixture is re-suspended from the filter and serial dilutions are plated onto a selective medium. Most often rifampicin resistance is used to select for the recipient. Depending on the aim of the study the medium used for selection of transconjugants contains additional antibiotics, heavy metal salts or xenobiotics. Only if the recipient captures an MGE which codes for respective resistances or confers degradative capabilities will it be able to form colonies on the plates. The biparental isolation of MGE depends on the stable replication of the MGE captured either by integration into the chromosome, or extra-chromosomally and the expression of respective phenotypes. MGE captured in a BPM can be conjugative or mobilisable plasmids or conjugative transposons (Smalla et al. 2000c). In a triparental mating (TPM; Fig. 13.2b), in addition to the recipient, a strain carrying a mobilisable plasmid, typically an IncQ-plasmid, is mixed with the bacterial fraction recovered from a soil sample. Selection for the recipient and resistances encoded by the small mobilisable plasmid will be made. In triparental matings, MGE can be isolated based on their mobilising activity. Although only a certain proportion of the recipients which obtained the mobilisable plasmid carry also the mobilising plasmid, the TPM approach still enables the capture of cryptic plasmids. Both tech-

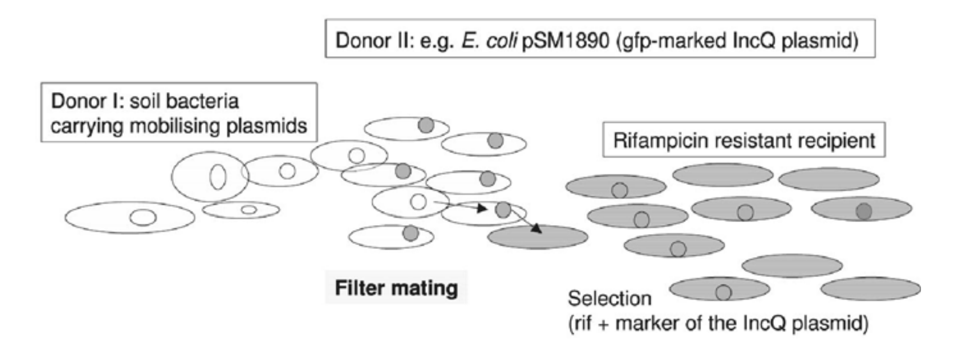
A. Biparental mating



➡ **Isolation of conjugative MGE independent from the culturability of their original host**

➡ **Isolation depends on the presence and expression markers used for selection**

B. Triparental mating



➡ **Isolation of MGE based on their mobilising capacity**

➡ **Isolation of MGE independent from the culturability of the original host and the expression of selectable markers**

Fig. 13.2. Capture of mobile genetic elements by exogenous isolation in biparental and triparental matings

niques were pioneered in the laboratory of J. Fry in Cardiff (Bale et al. 1988; Hill et al. 1992).

Exogenous isolation in BPM was used in several studies to capture MGE conferring mercury or antibiotic resistance or degradative activity to their respective recipients. Mercury resistance was used by Lilley et al. (1996) as an effective selective marker to exogenously isolate self-transferable Hg^r

plasmids from the phylloplane and rhizosphere of sugar beet in *P. putida* UWC1. Most of the Hg^r plasmids captured in BPM were between 250 and 380 kb in size and had a narrow host range. The presence of yet unidentified traits which seem to confer a fitness advantage to the host was demonstrated by Lilley and Bailey (1997a) when *P. fluorescens* SBW25 carrying the Hg^r plasmid pQBR103 (Tra^+, Hg^r, 330 kb) co-inoculated with plasmid-free *P. fluorescens* SBW25 on sugar beet seed was monitored. While initially a significant decline of the plasmid-carrying strain compared to the plasmid-free strain was detected, selection for the plasmid-carrying host was observed in the phyllosphere and rhizosphere of sugar beet plants approx. 100 days after planting.

Increased transfer frequencies in the rhizosphere of young wheat with increasing mercury pollution were observed by Smit et al. (1998) when studying the effect of mercury pollution in bulk and rhizosphere soils on the transfer frequencies in BPM of the bacterial soil community and the rif^r recipient strains *Enterobacter cloacae* BE1, *P. fluorescens* R2f, and *P. putida* UWC1. Characterisation of eight transconjugants revealed that all conjugative Hg^r plasmids also carried genes conferring resistance to copper. In addition to the Hg and Cu resistance, some of the plasmids also conferred resistance to streptomycin and chloramphenicol, and host range studies revealed a preference for hosts belonging to the β- and γ-proteobacteria (Smit et al. 1998). The preferential capture of Hg^r plasmids from the rhizosphere of young wheat roots reflects the dynamics of the plasmid hosts in response to plant growth. This observation is consistent with the study by Lilley and Bailey (1997b) who found that similar types of Hg^r plasmids were present only at a specific time during sugar beet plant development in three consecutive years. The effect of mercury addition on plasmid incidence and gene mobilising capacity was studied by Drønen et al. (1998, 1999) using the rif^r recipient strains *P. putida* UWC1, *E. coli* HB101, and *A. tumefaciens* UBAPF2. Addition of $HgCl_2$ to eight different soils resulted in increased colony-forming unit (cfu) counts of Hg^r bacteria as well as increased transfer frequencies and diversity of captured Hg^r plasmids. Self-transferable Hg^r plasmids were isolated from six out of eight mercury-contaminated soils and different types of Hg^r plasmids were captured in different recipients. Host range studies revealed that a considerable proportion of the isolated Hg^r plasmids had a broad host range. Conjugative Hg^r plasmids (pSB101–105) were also isolated by Schneiker et al. (2001) in BPM from the rhizosphere of alfalfa. The plasmids were obtained after filter-mating of the bacterial fraction recovered from alfalfa roots and the *gfp*-tagged *Sinorhizobium meliloti* strain FP2 as recipient. Plasmids pSB101 and pSB102 were repeatedly captured and thus seem to be abundant in the bacterial communities of the field site. The complete sequence of the 55,578 bp large Hg^r plasmid was recently published (Schneiker et al. 2001).

Top et al. (1995) have successfully modified the exogenous plasmid isolation approach to capture plasmids that carry genes coding for enzymes of the 2,4-dichlorophenoxyacetic acid (2,4-D) degradative pathway from soil microbial communities. Interestingly, in BPM with the rif^r *Ralstonia eutropha* JMP228 as recipient, plasmids conferring the ability to degrade 2,4-D were only obtained from those soils which were treated with 2,4-D for several years. In another study, Top et al. (1996) used the genetic complementation of *R. eutropha* carrying a pJP4 derivative with an inactivated *tfdA* gene to determine if certain soil bacteria contain self-transferable plasmids, which carry *tfdA*, a gene which may be unique and is required to initiate the 2,4-D metabolism. Again, complementation was only achieved with the microbial community from soils having a previous history of 2,4-D treatment. The authors suggest that HGT mediated by BHR plasmids might have contributed to the genetic mosaics seen for catabolic clusters.

A range of different self-transferable antibiotic resistance plasmids was captured, and, as observed also by Drønen et al. (1999), depending on the recipient strain used, different types of plasmids were isolated. The bacterial fraction recovered from soils treated with streptomycin or copper or sewage and from untreated soils served as donors of respective MGE in BPM with respective *gfp*-labelled recipient strains. While transferable resistance to streptomycin was easily captured from both bulk and rhizosphere bacteria in BPM, gentamicin resistances were obtained only from rhizosphere samples (Heuer et al. 2002; Van Overbeek et al. 2002).

Top et al. (1994) were the first to apply the TPM to isolate mobilising plasmids from different polluted soils and activated sludges. In this study *E. coli* CM330 with the IncQ plasmid pMOL155 (Tra$^-$, Mob$^+$) with a *czc* cassette carrying genes for resistance towards cadmium, zinc and cobalt which are not expressed in *E. coli* were used as donor of the mobilisable plasmid. The rif^r *R. eutropha* AE815 was used as recipient. Transfer of the pMOL155 would only occur to *R. eutropha*, where the *czc* gene cassette was expressed making a detection of transconjugants easily possible when mobilising plasmids were present. Since the IncQ plasmid pMOL155 is transferred from an *E. coli* strain (γ-subgroup) to an *R. eutropha* (β-subgroup) the involvement of BHR plasmids that mobilise this IncQ plasmid can be assumed. Mobilising plasmids were isolated from two of the four soils and from all sludge samples in TPM. Gene mobilising capacity of soils treated with manure was shown under field conditions by Götz and Smalla (1997). Mobilising plasmids were also isolated by Van Elsas et al. (1998) when bacterial communities obtained from the rhizosphere of young wheat plants served as donor in TPM according to the protocol described by Top et al. (1994). Plasmid pIPO2 was isolated in *R. eutropha* based on its mobilising capacity. This plasmid of a size of approx. 45 kbp was not related to any of the known BHR plasmids and no plasmid encoding selectable

traits such as antibiotic or heavy metal resistances were detected. Plasmid pIPO2 mobilised the IncQ plasmid pIE723 from *P. fluorescens* to indigenous bacteria in the wheat rhizosphere under field conditions and showed strikingly high retro-mobilisation frequencies (Szpirer et al. 1999). The complete sequence of this plasmid was recently determined and 42 ORFs were identified (Tauch et al. 2002). Most of the genes are predicted to be involved in plasmid replication, maintenance and transfer, and the overall organisation displayed striking similarities with pSB102 exogenously isolated from the rhizosphere of alfalfa (Schneiker et al. 2001) and pXF51 from the plant pathogen *Xylella fastidiosa* (Marques et al. 2001). The presence of long repetitive sequences in pSB102, pIPO2, and pXF51 seems to be characteristic for this new group of plasmids and differentiates them from other BHR plasmids such as IncP-1.

The wide use of the exogenous isolation approach has brought new insights into the horizontal gene pool of soil bacteria. Mobile genetic elements conferring selectable traits such as mercury or antibiotic resistance have been acquired from a wide range of environmental samples in diverse Gram-negative recipients functioning as a genetic sink. Capturing of degradative genes resident on MGE has also been demonstrated. Like in metagenomics (Schlosser and Handelsman 2003), exogenous isolation enables the capture of large DNA fragments, often complete operons from soil, without the need to cultivate the original host. In several studies increased transfer frequencies were observed when the environmental sample was previously exposed to pollutants. The major bias of the method is that it is still not working for Gram-positive bacteria.

13.4.3 PCR-Based Detection of MGE

Nucleic acids (NA) can be extracted from soil either directly or by cell extraction prior to the NA extraction and purification (see Chap. 3). The amount and quality of the NA obtained from 1 g of bulk or rhizosphere soil depends very much on the soil type, but differences have been also observed when different methods were compared with the same type of soil samples. Nowadays, many laboratories use one of the commercially available kits for direct DNA extraction from soil. When applied to a wide range of agricultural and forest soils, kits like BIO101 gave, after a harsh lysis step, good yields of PCR-amplifiable DNA which were comparable or higher than those obtained with the traditional protocol used in the past (Smalla et al. 1993). The DNA is used as target DNA in PCR reactions with primers targeting MGE-specific sequences (Greated and Thomas 1994; Götz et al. 1996; Smalla et al. 2000a; Krasowiak et al. 2002). This strategy was first

applied to study the presence of broad-host-range plasmids such as IncP1, IncW, IncN and IncQ in soils (Götz et al. 1996). The primers targeting genes involved in replication or transfer were designed based on the published sequences. The specificity of the primers was then tested with reference plasmids belonging to the different enteric Inc groups and a collection of plasmids which were assigned to the respective group under study by traditional Inc testing. When soil DNA is amplified with MGE-specific primers often no signals can be detected on the agarose gel. To increase the specificity and sensitivity of the detection, PCR amplicons are usually Southern blotted and hybridised with respective labelled probes (Smalla et al. 2000b,c). The intensity of the hybridisation signal gives a rather good semiquantitative estimate of BHR plasmid abundance. When strong PCR products can be detected already on the agarose gel (target sequences are at least 10^6 g^{-1} of soil) the PCR products can be cloned and sequenced.

13.5 Conclusions

Evidence is mounting that MGE play an important role in the evolution and adaptability of bacterial species. The rapid accumulation of bacterial genome sequences and the development of powerful tools has allowed, and will continue to allow, new insights into the horizontal gene pool, i.e. the horizontally transferred genetic modules and their mobile genetic vehicles. Identification of the flexible part of bacterial genomes – in contrast to that part which is shared by most strains of a species – will clarify what the cohesive factors forming bacterial species are. The capacity of soil microorganisms to sample the horizontal gene pool provides bacteria with the ability to rapidly adapt to ever-changing environments and is a major driving force of microbial diversity in soil.

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14 Bacterial Conjugation in Soil

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14.1 Introduction

Bacterial conjugation is a mechanism of horizontal gene transfer (HGT) by which donor cells transfer "protected" DNA (plasmid, conjugative transposon) via a conjugation bridge to compatible recipient cells (Koraimann 2004). The first exploratory studies on conjugation between bacteria under soil conditions were performed in the 1970s (Weinberg and Stotzky 1972; Graham and Istock 1978). Since then, an increasing number of studies have addressed conjugation in bulk soil as well as the rhizosphere (or phytosphere) of different plants using laboratory microcosm, greenhouse and field studies (reviewed in Van Elsas et al. 2000, 2003; Van Elsas and Bailey 2002). Most of these studies have focused on the effects of key environmental factors, such as temperature, moisture, nutrient availability, selection pressure and the presence of grazing protozoa or competing or antagonistic organisms, on the rates at which conjugative gene transfer takes place (Van Elsas et al. 2000; Timms-Wilson et al. 2001). The basic conjecture was that these factors were the key determinants of natural gene transfer rates and, if understood, could provide simple predictors of the frequency and likelihood of HGT mediated by conjugation in nature.

It is now generally accepted that conjugation represents a highly evolved mechanism of protected bacterial HGT (Trevors 1999; Van Elsas et al. 2003), which is of central importance in many habitats, including medical- and veterinary-related environments, sewage, manure, biosolids, plant surfaces and soil. The exchange of genetic material by conjugation in soil environments has strong relevance for the life strategies of bacteria in these habitats (Rensing et al. 2002). For example, conjugation may allow for the rapid generation of genetic and physiological diversity in a bacterial

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community in soil by mediating the spread of one or more conjugative plasmids throughout the population of compatible recipient cells (DiGiovanni et al. 1996; Dejonghe et al. 2000; Goris et al. 2002). Moreover, some plasmids can even stimulate the transfer of genes located on other plasmids or on the chromosome (Smit et al. 1993; Van Elsas et al. 1998). By transferring genetic elements such as whole plasmids or conjugative transposons, which can carry blocks of genes that encode related or unrelated functions, populations of bacteria in soil can make "quantum leaps" in their evolution and thus adapt to changing conditions in short periods of time (Fig. 14.1). The potential for such significant and abrupt changes may be relevant to the plant symbiosis (Sullivan and Ronson 1998) and pathogenicity conundrum. What causes a bacterium from soil to adapt to

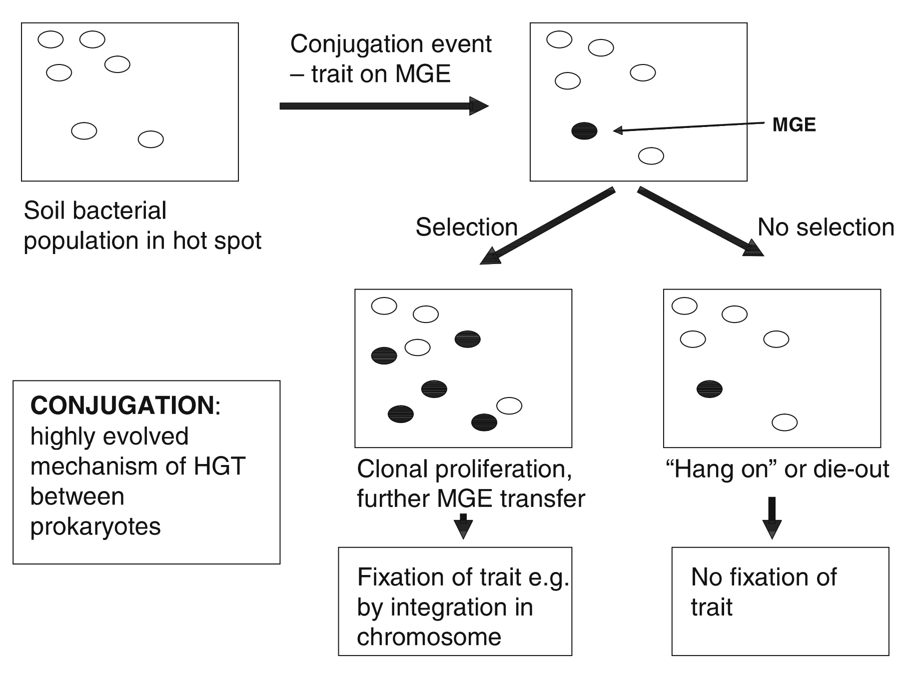


Fig. 14.1. Depiction of the impact of HGT via conjugation on bacterial communities in soil. Conjugative transfer is enhanced in so-called hot spots for conjugation, such as the rhizosphere, manure and the guts of soil invertebrates. An exogenous mobile genetic element (MGE) is transferred into one member of the community. In case of positive selection for a plasmid-encoded trait, this can lead to clonal outgrowth of the transconjugant, and/or further transfer of the MGE. Both processes enhance the chances of fixation of the novel trait in the population, e.g. by its integration into the chromosome. Instead of a single trait, a complex set of niche-adaptive traits can be transferred in one go, which can for instance result in a novel capacity for niche exploration. This has been called a quantum leap in evolution

plants and – eventually – evolve into a plant symbiont or even a pathogen? Genes involved in pathogenic or symbiontic interactions with plants are often located on genomic islands – denoted pathogenicity and/or symbiosis islands (Sullivan and Ronson 1998; Hacker and Carniel 2001). Furthermore, population studies have clearly indicated that the transfer of such islands by conjugation (or by transformation or transduction) may still play a role in current-day adaptations to the niche (Sullivan et al. 1996; Mohn-Zain et al. 2004). Recent comparisons of whole genome sequences seem to indicate that the transfer of genomic islands has been instrumental in bacterial speciation in response to differentiation of environmental niches (Hao and Golding 2004). However, an unresolved question is to what extent HGT processes play key roles in the current-day adaptation and evolution of, for instance, bacterial plant pathogens. We do not yet fully understand the details of these processes, but circumstantial evidence has already shown that gene transfer between bacteria, resulting in the transfer of genomic islands and plasmids that confer plant pathogenicity traits, is an obvious and important part of this problem. Thus, the environmental spread of plasmids or other mobile genetic elements that potentially carry plant or even human pathogenic or other undesirable functions should receive more research attention.

Furthermore, evidence is accruing that HGT by conjugation can be indispensable for a range of more generic processes in soil. Thus, genes involved in the more efficient utilisation of complex substrates, in the degradation of pollutants (Fulthorpe and Wyndham 1992) and in resistance to toxicants (Sundin et al. 1994) have been shown to spread in indigenous soil bacterial communities and to enhance the capabilities of these communities to thrive as well as survive under diverse adverse conditions. If these processes occur at relevant rates, the possibility that conjugation (and the other bacterial HGT mechanisms) could be employed to increase the potential for microbial adaptation to metabolise pollutants in contaminated environments via bioaugmentation should be considered (Rensing et al. 2002; Top et al. 2002a,b).

In spite of our increased understanding of HGT via conjugation in the soil environment, there still is a considerable lack of information on the intricacies of this process and on the ecological impact of HGT in natural bacterial assemblages. Questions remain with respect to in situ phenomena such as cell-to-cell signalling (triggering cell-to-cell contact leading to DNA transfer), as well as cell-to-mobile genetic element (MGE) contact. Furthermore, the varied ways in which these processes affect bacterial communities in the adaptation to their natural habitats and the dependence of all of these processes on the environment have received little attention. An in-depth understanding of in situ conjugation is thus needed to make progress in this area, and the further development of experimental approaches that allow

the direct monitoring of conjugative gene transfer among populations in their natural habitats is primordial for this.

A range of molecular biology and other techniques have been developed over the last decade that are applicable to microbes in natural settings (Akkermans et al. 1995; Kowalchuk et al. 2004). These methods have enabled researchers to investigate environmental gene transfer processes at much more refined levels of resolution than had been possible before. For instance, soil DNA/RNA-based assessments have provided a direct picture of the prevalence of specific MGE in soil. Comparison of the information contained in full sequences of plasmids (e.g. Tauch et al. 2002) or bacterial genomes (Salanoubat et al. 2002) provides insights into environmentally driven adaptive gene acquisitions and losses. Furthermore, when employed in DNA microarrays, genomic data allow the study of specific plasmid and/or host gene expression in relation to HGT in the natural habitat. In addition, the resolving power offered by reporter gene technology, especially that using the green fluorescent protein (GFP), has allowed detailed analyses of the patterns of gene transfer in microcolonies or biofilms to be made (e.g. Christensen et al. 1996, 1998). The impetus of these developments in fostering our understanding of environmental HGT and its impact on natural microbial communities is only beginning to emerge.

This chapter will focus on conjugative gene transfer in soil involving donor-to-introduced-recipient and donor-to-indigenous-bacteria matings. We briefly review the impact of key environmental factors (including selection), and then focus on the relevance of the genome sequencing revolution for our understanding of HGT processes in soil. In the studies discussed, plasmids have often been employed as these represent a class of readily transferable genetic elements (Smit et al. 1991, 1993; Richaume et al. 1992; Lilley et al. 1994; Van Elsas et al. 1998; Dejonghe et al. 2000). However, transfers of other genetic material, e.g. of conjugative transposons or of chromosomal genes (after mobilisation), can also be studied using similar approaches. Osborn and Böltner (2002) have recently described the commonalities in these and other MGE. The following list and Table 14.1 provide summaries of bacterial conjugation and the consequences of HGT processes in the soil environment, from (1) a mechanistic and (2) a genome fluidity and evolutionary perspective.

Characteristics of conjugation in soil summary:

- Highly evolved protected gene transfer mechanism – requires physical cell-to-cell contact via a conjugation bridge. The conjugation bridge can be supported on a surface such as soil particles, organic residues and plant surfaces.
- Frequency of conjugation (estimated using the ratio of transconjugant to donor cells). Range: $< 10^{-8}$–10^{-3}.

- Conjugation contributes to bacterial genetic diversity.
- Conjugation allows the rapid spread of plasmid DNA and hence is central to bacterial evolution.
- Allows hosts to receive useful traits such as antibiotic resistance, metal resistance and degradative genes.
- The outcome of gene transfer may lead to recombination events bringing useful genes together and the DNA repair mechanism may be commenced.
- Biochemical energy costs may be increased in cells maintaining acquired plasmids and expressed proteins.
- Upon death of plasmid-containing cells nutrients are provided to other soil microorganisms. At the same time DNA for transformation is provided (if the DNA is not completely digested by soil nuclease activity).

Table 14.1. Conjugation in soil – core genes versus auxiliary genes in soil bacteria (After Hacker and Carniel 2001)

Core (essential) gene pool	Auxiliary (fluid) gene pool
Chromosomal	On mobile genetic elements (MGE*)
Vertically transmitted	Horizontally transmitted
Genes part of bacterial cell cycle – partition upon cell division	Genes can be lost upon cell divisions
Slower evolution	Faster evolution
Mutations in key genes may or may not be beneficial	
Examples	**Examples**
Central biochemical pathways	Antibiotic resistance
DNA synthesis, replication	Metal resistance
Ribosomes	Degradation of chemicals
Cell envelope, membranes	Secondary metabolites
Cell division	Restriction-modification
Active transport mechanisms	Pathogenicity traits
	Transposases
	Symbiontic functions

*MGE: plasmids, conjugative transposons, bacteriophage, transposons

14.2 Experimental Approaches to Studying HGT via Conjugation

14.2.1 Conjugation Systems

Most experimental studies use conjugation systems composed of genetically marked donor and recipient bacteria, in which the donor bacteria carry self-transmissible and/or mobilisable plasmids. Self-transmissible plasmids can mediate their own transfer since they carry both an origin of transfer (*ori*T) and transfer (*tra*) genes. The *tra* genes are necessary for the formation of mating aggregates, nicking and unwinding of the plasmid DNA and transfer of single-stranded DNA from donor to recipient via the conjugation bridge. Plasmids are grouped in so-called incompatibility groups, and plasmids of some groups (for instance, IncP, IncN, IncW, IncX and as-yet-undefined groups exemplified by plasmid pIPO2) can mobilise non-self-transmissible (no *tra* genes) yet mobilisable plasmids (carrying the transfer origin *ori*T) to a suitable recipient strain (Thomas 1989, 2000; Clewell 1993). Transfer in the opposite direction has also been detected, i.e. certain plasmids were able to mobilise a plasmid into the (donor) strain which contained the self-transmissible plasmid (Mergeay et al. 1987). This process has been called retrotransfer or retromobilisation. The occurrence of retrotransfer might have implications for the transfer of recombinant DNA from introduced genetically modified bacteria in the environment to indigenous bacteria.

Conjugative gene transfer between bacteria in the soil environment can be studied in different ways (Smit and Van Elsas 1992; Timms-Wilson et al. 2001):

1. By introducing donor (containing a plasmid, conjugative transposon or other gene-mobilising agent) and/or recipient bacteria into the habitat (soil) and checking for gene transfer by selecting for the recipient and the selectable traits carried by the donor (Krasovsky and Stotzky 1987; Van Elsas et al. 1988; Richaume et al. 1992; Smit and Van Elsas 1992; Pukall et al. 1996; Götz and Smalla 1997; Van Elsas et al. 2003). The addition of donor and recipient cells at different ratios affects the frequencies (transconjugants/donor cells) of gene transfer. A ratio where there are more recipients than donors increases the probability that a donor cell will physically contact a recipient cell via the conjugation bridge. The rate of conjugation is dependent on the probability that donor and recipient cells are in close physical proximity (micrometer scale) for a conjugation bridge to successfully be established and mating not be interrupted by mechanical forces.

2. By introducing only donor bacteria in the habitat under study and assessing the transfer of genes into indigenous bacteria. For this purpose, a specific donor counterselection method is essential (Smit et al. 1991). In such experiments, the frequency of gene transfer is generally very low (e.g. 10–8 or below) and gene transfer may even be undetectable without selective force favouring the transconjugants.

3. By in situ observations, e.g. in biofilms, of the transfer of genetic material from donor to recipient bacteria, using an observable change of reporter gene expression (see Chaps. 16 and 17). This can be achieved by following the transfer of a plasmid or other genetic element with a represssed marker gene, for instance the gene encoding the green fluorescent protein, *gfp*, in the donor cell, into a recipient cell in which expression occurs (Christensen et al. 1996, 1998; Dahlberg et al. 1998; Hausner and Wuertz 1999). The green fluorescence can be easily detected using fluorometers, fluorescent plate readers or the green fluorescence of colony-forming units under UV light (Errampalli et al. 1999).

4. By analysis of sequences of genes encoding the same functions found in different genetic (chromosomal) backgrounds, and inferring potential gene transfer events. This is exclusively a retrospective process which will tell us about HGT over either shorter or longer periods of evolutionary time. It can be time-consuming and does not give an estimate of the transfer frequency, but it provides theoretical frameworks of how bacteria adapt to different environments that can be tested in the future.

14.2.2
Soil Microcosm Versus Field Studies

Soils are inherently complex, diverse and heterogeneous systems dominated by solid materials interspersed with liquid and gaseous phases. Experiments on conjugation executed directly in field soil are often plagued by variations in a range of abiotic factors that are difficult to control, or by problems of heterogeneity. Hence, in addition to a few field studies, investigators have largely relied on microcosms in their experimental approaches to monitor gene transfer (reviewed in Van Elsas et al. 2000, 2003; Van Elsas and Bailey 2002). Microcosms introduce some of the complexities that are present in the soil environment yet allow control over factors that are not controlled in studies in the field. In particular, the key abiotic soil factors moisture content and temperature are commonly tightly controlled in microcosm studies, whereas these will fluctuate in the field. Much of what we have learned so far on conjugation in soil systems has come from

microcosm studies and only incidentally have the data been validated by field work (Götz and Smalla 1997; Van Elsas et al. 1998; Goris et al. 2002).

14.2.3 What Did We Learn from Donor-to-Recipient Gene Transfer Studies?

Procedure

The traditional method to investigate conjugative gene transfer between introduced donor and recipient cells is relatively straightforward. Following incubation of donor and recipient cells in the soil or soil/plant system under study for a known period of time, cells are harvested and analysed for their genetic make-up. Thus, the detection of the gene transfer process, resulting in the formation of transconjugants, is commonly based on the use of selectable markers, often resistances to antibiotics or heavy metals carried by the mobile genetic elements transferred. However, several points need to be checked as they are key to the interpretation of the data. First, plasmid transfer may occur on the transconjugant-selective agar plates instead of in the habitat under study (Smit and Van Elsas 1990). Apparently, this so-called plate mating takes place depending on which donors or recipient bacteria are used. Before starting an experiment, one should verify if plate mating can occur, and, in addition, this aspect should be controlled for in the experiment. Using a *Pseudomonas fluorescens* strain as the plasmid donor, plate mating could be inhibited by using nalidixic acid as one of the antibiotics for donor counterselection, as a result of its DNA gyrase inhibitory action (Smit and Van Elsas 1990). Secondly, controls for mutation of donor and recipient bacteria should also be done to check for the occurrence of spontaneously resistant bacteria growing on the transconjugant-selective plates. Performing the experiment with the proper controls in place will allow the detection of "true" transconjugants as colonies on selective agar plates. These colonies will have to be subjected to a verification procedure and can then serve to investigate the genetic make-up following transfer.

Overview of Data

Numerous reports have documented the occurrence of conjugation between marked donor and recipient strains in soil and related environments (Krasovsky and Stotzky 1987; Van Elsas et al. 1987, 1988, 1998, 2000; Richaume et al. 1989, 1992; Smit et al. 1991, 1993; Wellington et al. 1992; Van Elsas and Bailey 2002). These experiments, in which donor bacteria (carrying transferable selectable markers) accompanied by recipient cells

were added to soil, collectively have provided direct evidence for the occurrence of conjugative gene transfer in soils. Successful conjugative gene transfer was shown to depend on the close physical contact between donor and recipient cells under favourable conditions of soil moisture, temperature, pH and nutrient availability. Soil factors such as organic matter content, clay content, water or nutrient availability were all shown to affect the gene transfer process to different extents (reviewed in Van Elsas et al. 2000). Briefly, and on the positive side, the presence in soil of nutrients, (montmorillonite) clay minerals, manure, plant roots and other surfaces and soil invertebrates, and the absence of a competing microflora, have all been shown to promote conjugative transfers (Van Elsas et al. 2000, 2003; Van Elsas and Bailey 2002). Transconjugants could be well detected after the addition of nutrients (Van Elsas et al. 1987) or after applying positive selection for plasmid-encoded functions (De Rore et al. 1994). Similarly, the presence of montmorillonite or bentonite clays (Van Elsas et al. 1987) resulted in enhanced conjugation rates. The presence of plant roots also resulted in enhanced numbers of transconjugants probably by enhancing cellular activity and cell-to-cell contacts (Van Elsas et al. 1988), although this effect might be plant species-dependent (Kroer et al. 1998; Sengeløv et al. 2001). Also, plasmid transfer was shown to occur frequently among epiphytic bacteria (Björklöf et al. 1995). In addition, the gut of the soil microarthropod *Folsomia candida* (Collembola) was shown to allow (intergeneric) transfer of conjugative and mobilisable plasmids harboured by *Escherichia coli*, and the earthworm *Lumbricus rubellus* stimulated the establishment of plasmids in the soil bacterial community (Hoffmann et al. 1998; Thimm et al. 2001). In another study, the presence of earthworms was shown to enhance donor-to-recipient contacts in the soil and, thus, stimulate conjugation (Daane et al. 1997). Recently, it has been found that the formation of a biofilm may enhance the efficiency with which gene transfer occurs (Hausner and Wuertz 1999; Prosser et al. 2000; Molin and Tolker-Nielsen 2003). This finding has stimulated research into the conjugative process in bacterial conglomerates (biofilms or microcolonies) occurring in specific niches in soil and the phytosphere (Lilley and Bailey 2002). Although these experiments are difficult and data are slow to appear, this is certainly a challenging area for future studies (Van Elsas et al. 2000, 2003; Van Elsas and Bailey 2002).

Summarising, all available data so far obtained using donor-to-recipient mating systems indicate that HGT via conjugation generally occurs at low frequencies in an undisturbed soil (see Sect. 14.1). Thus, soil, as a result of its grossly nutrient-limited and "static" character (resulting in spatial isolation), in the short time-span over which most experiments were completed, clearly had a barrier effect on the occurrence of matings between the bacterial assemblages. This barrier effect could be overcome under specific

favourable conditions which most likely resulted in, for instance, locally enhanced availability of nutrients, water conditions resulting in enhanced soil pore interconnectivity, and/or enhanced availability of colonisable surfaces. Such favourable conditions have likely increased bacterial growth and activities, resulting in localised high cell densities and optimal ratios of donor and recipient populations. Moreover, and last but not least, the occurrence of local or global positive selection pressures favouring the clonal development of transconjugants is a crucial factor that dictates the outcome of many conjugational HGT events (De Rore et al. 1994; Dejonghe et al. 2000; Rensing et al. 2002; Van Elsas et al. 2003).

14.2.4 What Did We Learn from Donor-to-Indigenous-Bacteria Transfer Studies?

Procedure

One of the main problems in studying conjugative gene transfer from introduced donor-to-indigenous bacteria is the application of sufficiently powerful donor counterselection. The donor cells inevitably carry the same selectable markers as the transconjugant cells and so usually overgrow the plates used for selection of the transconjugants since they are present in numbers greatly exceeding those of the putative transconjugants. Several possible methods have been suggested to eliminate the donor strain, and some have found wide application (Van Elsas et al. 2003):

1. The use of selectable markers that are repressed in the donor strain but not in potential indigenous transconjugants,
2. the use of an auxotrophic donor in conjunction with plating on minimal medium,
3. the use of a donor with an inducible/repressable host-killing gene,
4. the use of a donor which does not survive in the soil or related habitat after gene transfer has occurred (De Rore et al. 1994; Pukall et al. 1996), and
5. the use of a specific bacteriophage to lyse the donor cells following incubation in soil (Smit et al. 1991, 1993; Van Elsas et al. 1998).

Whereas most of the above approaches have been attempted, the bacteriophage-based approach has been successfully used in a range of studies on HGT via conjugation in the soil environment (Smit et al. 1991, 1993; Van Elsas et al. 1998). Crucial to the preciseness of these studies was the careful

assessment, in a one-by-one fashion, of the nature of the putative indigenous transconjugants found (Van Elsas et al. 1998). Major emphasis should be placed on removing false positives (e.g. donors that escaped donor counterselection, plate matants) as soon as possible from the analyses.

Overview of Data

Taken together, all studies on "donor-to-indigenous-bacteria" conjugation in the soil environment have largely confirmed the data obtained previously by using introduced donor-to-recipient mating systems (Smit et al. 1991, 1993; Van Elsas et al. 1998). Hence the same key factors (availability of nutrients, colonisable surfaces, selection for plasmid-encoded functions, or in general conditions that stimulate the local presence of dense bacterial communities) were shown to stimulate conjugative transfers of genetic material from introduced to indigenous bacteria (DiGiovanni et al. 1996; Van Elsas et al. 1998, 2000, 2003; Molbak et al. 2003), although the effects of these factors were not always dissected (Top et al. 2002a,b). The role of earthworms in promoting conjugative transfers was elucidated by Daane et al. (1996). Furthermore, Newby et al. (2000a,b) revealed that the apparent transfer of degradative plasmids was enhanced under selection for the plasmid-encoded functions. This was confirmed by Dejonghe et al. (2000) in a study on the transfer of plasmid pJP4 from *P. putida*. The strong positive effect of selection for plasmid-encoded functions on conjugative gene transfers was further highlighted in a theoretical framework by Rensing et al. (2002).

Interestingly, the types of organisms that had captured the transferred plasmids often belonged to typical r strategists, i.e. were members of bacterial groups that might be predicted to become active upon disturbance of the soil (Van Elsas et al. 1998; Goris et al. 2002). In other words, the measured transfers presumably occurred under the influence of the putative activation of possibly dormant inhabitants of the habitat under study. It still remains a challenge to distinguish this putative disturbance effect from the other factorial effects studied.

All studies on HGT in soil environments performed so far have been based on cultivation of transconjugants as well as donors and recipients. However, the culturable fraction of bacteria is often below 1 to maximally 10% of the total numbers of bacteria determined by direct microscopic counts. Culturing might also be biased due to the viable but non-culturable (VBNC) state that some bacteria may enter. Hence, the significance of all HGT studies performed so far (that relied on selective growth) for transfers to the whole community might be questioned. To bring this field forward, new techniques that do not exclusively rely on culturability are needed. As outlined below, such approaches may refine our view of environmental

gene transfer processes and ultimately will provide better estimates of the rates and extent of gene transfer in natural environments.

14.3 Conjugative Transfer to Total (Potentially Non-culturable) Bacteria

HGT into the non-culturable fractions of bacterial communities in soil and related environments can be assessed by two divergent, and possibly complementary, approaches. First, as outlined below, using marker gene technology in an introduced donor strain, in situ observations can be made with respect to the transfer of mobilisable DNA. Similarly, transfer into an introduced marked recipient can be measured (e.g. Lilley et al. 1994). Second, taking genomic sequence information on board, an assessment can be made of similarities between gene sequences in different bacteria dwelling in the soil habitat. This approach can either be based on bacterial isolates, or on a soil DNA-based metagenomics approach, in which there is no need to culture the underlying organisms. The former method still relies on cultivation, but can be used in a comparative fashion with the latter. That is, information on both sequence and linkage of genes obtained from isolates can be coupled to that obtained directly from soil.

In the following, we will discuss what can be learned from in situ gene transfer studies as well as from comparisons of genomes of cultured bacteria. We will then briefly address how soil metagenomics can assist us in understanding HGT processes in soil.

14.3.1 What Did We Learn from Studies on In Situ Conjugative Gene Transfer?

Procedure

Assessment of in situ gene transfer can be achieved with the aid of *gfp*, the gene encoding the green fluorescent protein (GFP), as a marker gene. The use of the GFP – from the jellyfish, *Aequorea victoria* – offers key advantages. First, GFP is intrinsically fluorescent. The chromophore is post-translationally formed in the presence of oxygen from serine-tyrosine-glycine at amino acids 65–67. Wild-type GFP is excited by blue light at 395 nm and emits green light at 509 nm. The fluorescence is stable and can be monitored non-invasively in living cells in real time. Expression of GFP was used as the discerning criterion in a conjugation study, by inserting the *gfp* gene downstream of a promoter not recognised by the donor RNA

polymerases, but which could be transcribed in recipient cells. Thus, GFP expression that occurred following transfer to a suitable recipient could be monitored by epifluorescence microscopy. The concept of using a conjugative donor bacterium in which the plasmid-localised *gfp* gene is repressed, whereas it can be easily expressed in other (added or indigenous) strains following a transfer out of the donor, has found wide acceptance (Christensen et al. 1996, 1998; Dahlberg et al. 1998). Hausner and Wuertz (1999) used an alternative fluorescent marker (TRITC) as a probe to distinguish between transconjugants and donor cells.

Overview of Data

As outlined, several recent studies have used GFP-based methods to study conjugative gene transfer without the need for culturing, and these studies have allowed a different perspective of conjugative processes in natural habitats (Christensen et al. 1996, 1998; Dahlberg et al. 1998; Normander et al. 1998). Normander et al. (1998) studied the transfer of a *gfp*-marked conjugative plasmid out of a *Pseudomonas putida* KT2442 donor strain. The donor contained a chromosomal *lacI*^{Q1} insertion and the LacI protein repressed transcription from a modified *lac* promoter upstream of the *gfp* gene on the conjugative plasmid. When the plasmid was transferred to indigenous bacteria on plants, repression was relieved in the novel (*lacI* minus) hosts and green fluorescent transconjugant cells could be detected by epifluorescence microscopy. Similar studies have been successfully performed in model biofilm structures. These studies have depicted biofilms as matrices of cells between which conjugative processes could easily take place, albeit not without limitations determined by the local physiology of the cells in the biofilm (Christensen et al. 1996; 1998, Dahlberg et al. 1998). In the light of our knowledge about the physical structure of bacterial communities in soil, there are major questions as to the extent to which such biofilms can and will occur in soil and related systems such as the rhizosphere and rhizoplane. Only with more detailed knowledge of biofilm (or, for that matter, microcolony) incidence in soil and related systems will we be able to dissect the conjugative process in this habitat at any greater level of detail.

14.3.2 What Did We Learn from Sequence Analyses of Soil/Phytosphere Bacteria?

Procedure

Comparative genomics is the process by which the gene content and organisation of fully sequenced genomes are compared. These comparisons are

usually among closely related bacteria from different habitats (e.g. within a species or genus where multiple genome sequences have been determined) or more distantly related bacteria that share a common habitat. Both are intended to identify niche-adaptive traits (by identifying different and shared traits, respectively) and require substantial ecological data for intelligent interpretation of intra- and intergenomic gene distribution patterns.

Overview of Data

Genomic comparisons have provided substantial insights into the impact of HGT on bacterial adaptation. In particular, HGT has been implied in the ability to make environmentally adaptive quantum leaps in metabolic (e.g. the acquisition of catabolic capacities) or functional (acquisition of virulence factors and symbiotic genes) potential. In part, this ability may stem from the highly dynamic nature of the horizontal gene pool, with frequent re-assortment of existing, functional gene cassettes to generate novel combinations of genes (e.g. giving rise to multi-drug resistance; Toussaint and Merlin 2002), and intergenic domain re-assortments to generate novel functional capacities, such as the degradation of xenobiotics (Omelchenko et al. 2003; Lopez 2004).

The number of whole bacterial genome sequences in public databases is continuing to grow apace, as are whole plasmid sequences. Both genome and plasmid sequence databases are still dominated by sequences of organisms of medical importance, but this bias is currently being addressed, as increasing numbers of genomes and plasmids of soil and aquatic bacteria are being sequenced. Comparative studies help us to reveal patterns of genome architecture and composition, which are indicative of the processes – including gene transfers, gene duplications and gene losses – that have contributed to bacterial adaptation. Most researchers agree that a conserved core of essential genes exists among most bacteria (Feil 2004), and that, beyond this core, an auxiliary gene set of varying nature is present. There appears to be a correlation between genome size and habitat. Bacteria that colonise homogeneous and/or stable environments (intracellular pathogens/symbionts and commensals of animals) have smaller genomes than those inhabiting heterogeneous and/or variable environments such as soil (Doolittle 2002; Ragan and Charlebois 2002). For example, among the proteobacteria, the genomes (chromosome plus plasmids) of the soil-dwelling plant pathogen *Ralstonia solanacearum* (5.8 Mbp) and free-living *Chromobacterium violaceum* (4.8 Mbp) are larger than those of the pathogenic *Neisseria meningitidis* (ca. 2.2 Mbp). Similarly, the free-living facultative α-proteobacterial symbionts of legumes *Sinorhizobium meliloti* (6.8 Mbp), *Mesorhizobium loti* (7.6 Mb) and *Bradyrhizobium*

japonicum (9.1 Mbp) have considerably larger genomes than their pathogenic relatives, i.e. the brucellas (< 3.5 Mbp) and rickettsias (< 1.3 Mbp; http://www.ncbi.nlm.nih.gov/genomes/static/eub.html). This pattern is also apparent among genomes of other groups of bacteria, including the high G+C% Gram-positive actinobacteria and archaea. The genome of the free-living and environmentally widely distributed species *Methanosarcina acetivorans* is by far the largest archaeal genome (5.75 Mbp) sequenced to date (Galagan et al. 2002). By comparison, the free-living archeaon *Picophilus torridus* that thrives in restricted and globally rare but constant conditions (optimally at 60 °C and pH 0.7) has a considerably smaller genome (1.55 Mbp; Fütterer et al. 2004). As a consequence of these observations, the large genome sizes are hypothesised to allow greater genetic flexibility in the adaptation to dynamic and heterogeneous habitats (Galagan et al. 2002; Salanoubat et al. 2002; Brazilian National Genome Project Consortium 2003).

Such observations raise the questions of "how have these large genomes been assembled and what is the impact of HGT processes such as conjugation". There is ample evidence that plasmid or genomic island exchanges have contributed to genome evolution. Several environmental species have megaplasmids or secondary chromosomes, none of which have chromosome-like replication systems and probably evolved from plasmids (e.g. Salanoubat et al. 2002). Genomes, in particular the larger ones, have further been shaped by gene duplications, as evident from the large numbers of homologues within certain gene families, including membrane transporters, chemosensors, regulators and extended catabolic capacities (Galibert et al. 2001; Salanoubat et al. 2002, Stover et al. 2000).

What are the underlying evolutionary processes that shape genome size? Is it likely that bacterial genomes have acquired genes through HGT, and differentially lost genes of no environmental benefit throughout evolution, giving rise to the bacterial lineages in existence today? The latter has been hypothesised for genomes of bacteria adapted to stable habitats such as intracellular pathogens (Gil et al. 2003; Van Ham et al. 2003), including intracellular plant pathogens (Oshima et al. 2004). An alternative hypothesis explaining the occurrence of large genome sizes is that gene duplications and divergent evolution of paralogues (duplicated genes) to achieve functional diversity, rather than HGT, may have contributed to genome expansions. Such gene duplications may have been facilitated by the presence of low-copy-number plasmids able to stably replicate and transfer extensive regions of duplicated DNA. To date, there is insufficient evidence to confirm or refute these ideas. However, evidence from the multiple sequenced species genomes (e.g. *E. coli*) support the hypothesis that horizontal gene acquisitions have contributed significantly to genome expansions as well as to adaptations to the niche (Perna et al. 2002). These ideas are also

supported by comparisons of the genome sequences of closely related bacteria – indicating that some gene sequences have distinct histories that are only explicable by invoking HGT (Hao and Golding 2004). For instance, the non-orthologous sequences (not inherited vertically) within the genomes of *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* were especially associated with the non-chromosomal replicons (Wood et al. 2001). Conversely, the commonality of habitat-responsive genes among these and other plant-associated species such as *R. solanacearum*, *Xanthomonas axonopodis* pv. *citri*, *X. campestris* pv. *campestris* and *Xylella fastidiosa* also indicates HGT is associated with adaptation to the environment (Da Silva et al. 2002; Salanoubat et al. 2002). The genome of the metal-reducing δ-proteobacterium *Geobacter sulfurreducens* contains metal-ion-reducing genes whose closest homologues are found in the γ-proteobacterium *Shewanella oneidensis* (Methé et al. 2003), again indicative of horizontal acquisition by one or the other lineage.

Further evidence to support the influence of HGT among environmental bacteria derives from the analyses of Tyson et al. (2004) who identified mosaic sequence reads of the *Ferroplasma* type II composite genome assembled directly from environmental libraries produced from acid mine drainage. These findings are indicative of substantial amounts of DNA transfer and recombination within the *Ferroplasma* type II species complex existing within the system studied. However, these intra-species exchanges have not brought about gross changes in gene content, such as those that might result from inter-species plasmid or genomic island acquisitions. This study clearly demonstrates that horizontal gene exchange within natural microbial populations can generate genome mosaics, as do studies of chromosomal loci in *Pseudomonas aeruginosa* environmental isolates (e.g. Kiewitz and Tümmler 2000).

Population and genomic studies thus confirm that many niche-adaptive traits, such as the pathogenicity determinants of *Pseudomonas* plant pathogens (Arnold et al. 2003), the symbiotic island of *M. loti* and symbiotic plasmids of other rhizobia, are part of the horizontal gene pool (Sullivan and Ronson 1998). These traits are carried on (self-) mobilisable elements with discontinuous distributions within and among species and distinct genetic signatures (of which anomalous G+C content is most widely cited). Surveys of plasmid and plasmid-borne gene sequence diversity among plant-associated species indicate extensive plasmid/gene transfers and recombination to generate mosaics of different related alleles (Rigottier-Gois et al. 1998; Turner et al. 2002).

On the basis of these cultivation-dependent data, we conclude that horizontal gene transfers among soil bacteria are potentially widespread, and that these are contributing to bacterial evolution and adaptation in natural soil and phytosphere systems. However, the majority of soil bacteria is – as

yet – unculturable, and the only practical way to extend our understanding of HGT processes to these bacteria is via a metagenomics approach. Using shotgun cloning of soil DNA followed by high-throughput sequencing and intelligent sorting and interpretation of the data, metagenomics will enable us to dissect the genetic linkages of selected traits under study. For instance, studying the linkage of specific genes from the auxiliary pool to phylogenetic markers should allow the visualisation of the extent these genes have spread through the soil metapopulation. Whereas this approach is by no means simple or fast, it is very feasible given the sheer power of the high-throughput sequencing machinery that is currently available worldwide. Thus, the extent to which the emerging picture of high mobility of genes from the auxiliary gene pool will hold true is in principle assessable using this advanced toolbox.

14.4 Conclusions

To elucidate the outcome of conjugation in soil and the phytosphere, data on the ecological factors that affect transfer, such as soil moisture, temperature, pH, nutrient status, cell-to-cell ratio, mating contact time and rate, and selective pressure, should be supplemented with other measurements at more refined levels. These include assessments of how bacterial host cells sense, and respond to, environmental changes, how they interact with other similar or dissimilar cells (by mechanisms such as signalling, competition and antagonism), and how these community-level processes affect HGT by conjugation. In addition, there is a need to identify and define the traits that impact on the fitness of the hosts that carry genes of the horizontal gene pool. This potential is largely cryptic and may pertain to functions that are difficult to mimic in the laboratory, yet are of immense importance for the survival of the host and the horizontally acquired DNA alike. It is likely that the current-day combination of molecular biology, genomics and ecology will reveal many of the best-kept secrets of the horizontally acquired DNA. For example, novel gene clusters containing open reading frames (ORFs) which probably encode small proteins of unknown function have been described on several plasmids and chromosomes (e.g. Tauch et al. 2002). These proteins are hypothesised to play a role in the cell-to-cell contact mechanism leading to HGT in the natural habitat. Alternatively, they may function in the interaction between the plasmid host and the plant rhizosphere as its preferred niche. The carriage of such a plasmid may thus be advantageous to the host dwelling in this natural setting. This contention is supported by the observations that the carriage or transfer of large plasmids by *Pseudomonas* spp. appeared to be advantageous only

at a very particular period during growth of the host plant (Lilley and Bailey 1997). Hence, carriage of these plasmids and expression of plasmid functions may promote the fitness of the host only under certain conditions. Thirdly, plasmid pSym carriage was shown to be a disadvantage in rhizobia surviving in bulk soil (no rhizosphere present) in the field, yet to enhance fitness in the rhizosphere (Sullivan and Ronson 1998). This pointed to a function other than just nodulating capacity as the mechanism behind the fitness-enhancing trait. These three observations provide interesting leads for future fundamental research on the ecology of gene transfer. In particular, there seems to be a much more intimate relationship, in ecological terms, between transfer and host fitness than previously thought. In other words, the mechanism and triggering of plasmid transfer may have very intricate links to host fitness and/or the sensing of conditions in the natural habitat.

Finally, it is axiomatic that the traits involved in successful HGT events, that (positively) affect host fitness, are of ecological and evolutionary importance. Hence, we need to better understand the ecological aspects of HGT, particularly in respect of the questions what types of conditions, compounds or signals stimulate HGT and result in stable incorporation of novel traits into a new host. Furthermore, resolving those environmental conditions that affect cellular metabolism and cell-to-cell contacts remains the key focal point for many researchers. In the future, the application of novel high-throughput molecular tools, such as DNA arrays, will potentially allow the study of in situ expression of transfer-related genes. It is hoped that this will provide new knowledge on the factors in natural habitats that trigger events leading to cell-to-cell contact (mating pair formation) and HGT. Alternatively, the use of reporter genes downstream of promoters involved in triggering the expression of these genes can be used.

To understand the impact HGT has had, and still has, on the evolution of soil- and plant-associated bacteria, comparative genomics should focus on sequences of interest that are present in organisms that share a common niche versus those that do not share the niche. For obvious reasons, niche sharing, as opposed to spatial isolation, offers the greatest chance for organisms to also share genes. It would be of great interest to assess to what extent organisms in shared niches actually use similar systems, introduced by HGT, in their striving for optimal adaptation to, and colonisation of the niche, and how the forces of selection act on these potential competitors. Are traits with clear and strong adaptive value indeed highly mobile and can they be shared by various organisms occupying a specific niche in soil? To what extent does this phenomenon serve the genes versus the organisms? There are several questions behind adaptive traits being shared within the niche which are not easily answered on theoretical grounds.

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15 Horizontal Gene Transfer by Natural Transformation in Soil Environment

Anne Mercier, Elisabeth Kay, Pascal Simonet

15.1 Introduction

Based on recent data from complete bacterial genome sequences, horizontal gene transfer (HGT) would have been a major evolutive force in bacterial evolution. However, even if some bacteria exhibit a particularly high level of laterally acquired genetic information (Ziebuhr et al. 1999; Ochman et al. 2000; Gupta and Maiden 2001; Jain et al. 2002), other bacterial species have developed efficient mechanisms to limit the rate of exogenous DNA acquisition in order to better stabilise their genome (Lorenz and Wackernagel 1994; Matic et al. 1996; Nielsen 1998). The balance between HGT, which confers specific capabilities and gene conservation, which aids in bacterial classification, demonstrates the importance of understanding bacterial plasticity (Arber 2000; Woese 2000; Brochier et al. 2002; Daubin et al. 2002; Brown 2003; Kurland et al. 2003). Moreover, this understanding has direct applications in assessing the risks related to the use of genetically modified organisms such as plants, which could become a possible source of transforming DNA for environmental bacteria (Nielsen et al. 1998; Bertolla and Simonet 1999). Interest in the fate of transgenes was mainly due to persistence of antibiotic resistance genes that were initially used as selective markers for plant construction (Flavell et al. 1992; Scutt et al. 2002), in plant genomes and its potential transfer to human pathogenic bacteria (Dröge et al. 1998; Normark and Normark 2002). The bacterial origin of these new plant genes could help them overcome the bacterial genetic barriers that had prevented all but a few rare plant genes being transferred from plants to bacteria (Brown and Doolittle 1999; Brinkman et al. 2002). In addition, the risk of transgene transfer would increase when transplastomic plants are considered (presence of the transgene in the chloroplast genome) due to the higher copy number that is increased from 10 in traditional and nuclear-modified transgenic plants to more than 10,000 for transplastomic plants (Daniell et al. 1998).

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HGT in bacteria has been investigated at various levels including in silico analyses, with appropriate bioinformatics tools to infer transferred genes in complete genome sequences (Eisen 2000; Garcia-Vallvé et al. 2000, 2003; Brown 2003; Daubin et al. 2003). Gene-transfer mechanisms including natural transformation, conjugation and transduction have also been studied at the genetics and physiological levels on some bacterial models (Amabile-Cuevas and Chicurel 1992; Lorenz and Wackernagel 1994; Dubnau 1999). Microcosms and open environment based experiments have been developed to determine the occurrence of gene transfer in situ and its potential impact on microbial community and ecosystems (Lorenz and Wackernagel 1994; Nielsen et al. 1998; Davison 1999; Dröge et al. 1999; Paul 1999; Timms-Wilson et al. 2002).

Critical questions related to assessing the gene-transfer events between bacteria and the risk in using transgenic organisms include determining where gene transfer could occur in the environment, which molecular mechanisms are involved, and how they are regulated in situ by the bacteria themselves and also by environmental factors.

15.2 Mechanisms of Horizontal Gene Transfer

In bacteria, three mechanisms, including conjugation, transduction and natural transformation, have been reported to be involved in HGT (Yin and Stotzky 1997; Ochman et al. 2000). Conjugation (see Chap. 14) and transduction promote transfer of genetic elements like plasmids, bacteriophages, genetic islands, transposons and integrons. These accessory elements are important vectors for the spread of genes coding for pathogenic traits, resistance to heavy metals or antibiotics among bacteria (Dobrindt and Hacker 1999, 2001; Rowe-Magnus and Mazel 2001). Some of these elements, such as conjugative plasmids or transposons, contain all or most of the genes required to promote their own transfer to bacteria belonging to the same or different species.

In contrast, natural transformation, which is the active uptake by competent bacteria of extracellular DNA present in their surrounding environment, requires coordinated functions encoded by a set of genes distributed throughout the genome of recipient bacteria (Lorenz and Wackernagel 1994; Friedrich et al. 2001). Products of these genes are involved in the four interconnected steps of the natural transformation process, including competence development, DNA binding, DNA uptake and the inheritable integration of the incoming DNA (Lorenz and Wackernagel 1994; Dubnau 1999; Chen and Dubnau 2003). Natural transformation-mediated gene acquisition and illegitimate or homologous recombination-based genomic

integration of incoming DNA into the host genome were demonstrated to occur both among bacteria belonging to the same species and to phylogenetically less related taxa (Lorenz and Wackernagel 1994; Kriz et al. 1999; Sikorski et al. 2002).

While transduction and natural transformation were often regarded as ecologically irrelevant in soil environments (Davison 1999), conjugation was considered as the most efficient gene-transfer mechanism occurring in situ. It was also the easiest mechanism to investigate by the use of micro- or mesocosms in which conjugative plasmid transfer frequencies between donor and recipient bacteria could be determined (Sengelov et al. 2000, 2001; de Lipthay et al. 2001; Desaint et al. 2003). According to these studies, several soil environments were recognised as being "hot spots" for HGT in comparison to the bulk soil including the rhizosphere (soils surrounding plant roots), the spermosphere (soils surrounding germinated seeds) and, finally, the "residuesphere" (interface between decaying plant material and soil matrix; Van Elsas et al. 1988; Troxler et al. 1997; Sengelov et al. 2000, 2001; de Lipthay et al. 2001; Nielsen et al. 2001).

15.3 In Situ Regulation of Natural Transformation in Bacteria

Several processes related to the bacteria themselves or to their environment appear to limit the occurrence of transformation-mediated genetic transfer. The number of bacteria described as being naturally transformable is relatively low, thus limiting the potential impact of the phenomenon on the microbial community (Lorenz and Wackernagel 1994; Davison 1999). In transformable bacteria, the uptake of DNA is submitted to a series of filters by several successive mechanisms that decrease success rates. In some competent bacteria, transformation is restricted to DNA from the same species, i.e. to DNA that contains species-specific "uptake signal sequences" required in order that the DNA binds to the cell wall prior to uptake (Smith et al. 1999). Although restriction and modification systems recognise and cleave double-stranded DNA, they can also degrade single-stranded DNA produced during natural transformation (Ando et al. 2000; Aras et al. 2002; Berndt et al. 2003). The last step of genetic transformation is the stable inheritance of the entering DNA either by autonomous replication for a plasmid molecule or by recombination-mediated genomic integration of linear DNA (de Vries and Wackernagel 2002, Meier and Wackernagel 2003a).

Efficiency of integration by homologous recombination depends on the size of the DNA and sequence divergence between donor and recipient DNA regions (Strätz et al. 1996; Demanèche et al. 2002). Two main antagonistic systems, the mismatch repair system (MRS) and the SOS system, are

involved in the regulation of heterologous DNA integration (Matic et al. 1996; Taddei et al. 1997; Nielsen 1998; Young and Ornston 2001). Bacterial genome stability depends on the expression level of these two systems with the MRS acting as a strong but flexible genetic barrier to prevent any uncontrolled genetic drift due to integration of heterologous DNA.

Occurrence of natural transformation in the environment was also questioned due to adverse conditions encountered by DNA and bacteria in situ. Soil conditions were thought to be physically, chemically and enzymatically incompatible with long-term persistence of free DNA. In addition, several studies failed to demonstrate that bacteria could develop competence in situ (see below).

15.4
Natural Transformation: An Unexpected Widespread Gene-Transfer Mechanism in Bacteria?

Extracellular DNA present in the vicinity of bacteria is used by most bacteria in situ as a source of carbon and energy for supporting bacterial growth. In addition this DNA can also be used for genome evolution relevant functions such as DNA repair (Michod et al. 1988; Mongold 1992; Redfield 1993; Solomon and Grossman 1996; Redfield et al. 1997; Finkel and Kolter 2001). Interestingly, DNA consumption in non-competent bacteria such as *Escherichia coli* and some other proteobacteria involves enzymes that are homologous to proteins involved in the DNA-uptake function of competence in naturally transformable bacteria (Finkel and Kolter 2001). The evolutive interest in these functions is further supported by the detection of highly homologous modules composed of DNA-uptake genes in distantly related bacteria suggesting possible acquisition of these modules by HGT and their integration at different genomic loci in the host bacterial genomes (Friedrich et al. 2001).

The list of bacteria known to possess a genetically encoded natural transformation machinery seems very limited in comparison to the huge bacterial diversity. However, these bacteria encompass a wide range of physiological capabilities: photolithotrophy, chemolithotrophy, heterotrophy, and methylotrophy (Table 15.1). While most of the known naturally transformable bacteria are mesophiles, some are thermophiles, and the list includes saprophyte bacteria as well as plant and animal symbionts and pathogens (Lorenz and Wackernagel 1994). However, the transformation potential of soil bacteria remains almost totally unknown; most isolates from worldwide culture collections having never been tested for competence development under in vitro or in situ conditions (Demanèche et al. 2001c). Moreover, the competence development property is not sys-

Table 15.1. Non-exhaustive list of naturally transformable bacteria isolated from soil environment

Species present in soil environment		References
Natural transformable bacteria (competence regime identified)		
Photolithotrophic	*Synechocystis* sp. PCC 6803[a]	Yoshihara et al. 2001
Chemolithotrophic	*Thiobacillus thiopurans*[b]	Yankofsky et al. 1983
Heterotrophic	*Acinetobacter calcoaceticus*[a]	Palmen et al. 1993
	Azotobacter vinelandii[b]	Page and Grant 1987
	Bacillus subtilis[a]	Dubnau 1991
	Deinococcus radiodurans	Fuchs et al. 1994
	Mycobacterium smegmatis	Norgard and Imaeda 1978; Bhatt et al. 2002
	Pseudomonas stutzeri[a]	Carlson et al. 1983; Meier et al. 2002
	Ralstonia solanacearum	Bertolla et al. 1997
	Thermus thermophilus[a] (and *Thermus* spp.)	Koyama et al. 1986; Friedrich et al. 2001, 2002
Methylotropic	*Methylobacterium organophilum*[b]	O'Connor et al. 1977
Archaebacteria	*Methanobacterium thermoautotrophicum*[b]	Worrell et al. 1988
Clinical pathogenic spp.	*Campylobacter jejuni*[a]	Wiesner et al. 2003
	Campylobacter coli	Wang and Taylor 1990
	Helicobacter pylori[a]	Hofreuter et al. 2000; Smeets and Kusters 2002
	Legionella pneumophila[a] (serogroup 1)	Stone and Kwaik 1999
	Moraxella spp.[a]	Juni et al. 1988; Luke et al. 2004
Suspected natural transformable bacteria (competence regime non-identified)		
	Agrobacterium tumefaciens[d]	Demanèche et al. 2001c
	Escherichia coli[c]	Claverys and Martin 2003
	Lactococcus lactis[c]	Bolotin et al. 2001
	Listeria monocytogenes[c]	Claverys and Martin 2003
	Pseudomonas fluorescens[d]	Demanèche et al. 2001c

[a] Competence regime identified and completed by molecular analyses of the natural transformation machinery
[b] No recent publication about natural transformation of the species considered
[c] In silico detection of a natural transformation machinery
[d] In situ evidence of natural transformation capability

tematically shared by all representatives belonging to the same species, and transformation frequencies can vary up to four orders of magnitude among transformable isolates of the same bacterial species (Sikorski et al. 2002).

The number of transformable bacteria (44) proposed 10 years ago by Lorenz and Wackernagel (1994) has increased significantly with data from

complete genome sequences and experimental studies (Claverys and Martin 2003; Davidsen et al. 2004). Genes encoding homologues of the DNA-uptake machinery components were detected in numerous bacteria, including *Lactococcus lactis*, *Streptococcus pyogenes*, and *E. coli*, for which natural competence development had not been reported previously (Bolotin et al. 2001; Claverys and Martin 2003). Thus, these bacteria might also undergo an active and genetically encoded transformation process.

In silico detection of competence sequences in bacterial genomes motivates new experimental studies in order to determine if the related genes are still functional and which conditions are necessary for their expression (Claverys and Martin 2003). Such studies could rapidly lead to a significant extension of the list of naturally transformable bacteria and to a better understanding about genesis, evolution and the spread of the genetic transformation mechanism among bacteria and its role in adaptation.

15.5 Bacterial Competence Development in Soil

The level to which a bacterial population is modified by natural transformation depends on natural transformation frequencies directly related to the ability of bacteria to take up DNA. Natural transformation has been detected in various environments including seawater and marine sediments (Paul et al. 1991; Paul 1999), plant tissues (Bertolla et al. 1999; Kay et al. 2003) and soil (Lorenz and Wackernagel 1994; Nielsen et al. 1997, 2000a,b; Dröge et al. 1999). Most of these studies included the use of naturally transformable bacteria inoculated in microcosms or mesocosms with the objective to simulate natural conditions demonstrating that competent bacteria can take up extracellular DNA in situ.

Bacteria such as *Bacillus subtilis*, *Acinetobacter* spp. and *Pseudomonas stutzeri* were selected as models on the basis of criteria related to their transformation potential in vitro (Lorenz and Wackernagel 1994; Kay et al. 2002a,b; de Vries et al. 2001).

Naturally transformable bacteria exhibit significant differences in competence development (Solomon and Grossman 1996). Competence is expressed constitutively in few bacteria but this state is more often related to an active physiological state corresponding to the exponential growth phase under in vitro conditions (Lorenz and Wackernagel 1994; Solomon and Grossman 1996). Other bacteria require accumulation of a competence factor in the surrounding medium (Solomon and Grossman 1996). For those bacteria, only cells at the stationary phase become competent (Lorenz and Wackernagel 1994). These differences between bacteria can be

explained by their need to adapt to specific environmental niches depending on their location in soil microhabitats.

The biotic and abiotic conditions of the different soil microhabitats, such as competition with other bacteria, predation, pH, moisture level, concentration of mono- and bivalent cations, and nutrient input, are known to affect the natural transformation process (Timms-Wilson et al. 2002). Soils are often described as being more refractory than marine environments for bacterial competence development due to the matrix heterogeneity and the oligotrophic conditions which lead bacteria to survive in a state of dormancy (Lorenz and Wackernagel 1994; Nielsen et al. 1997). For example, *Acinetobacter* sp. strain BD413, which exhibits a particularly high transformation frequency in vitro (Palmen et al. 1993), did not develop competence naturally in soil (Nielsen et al. 1997). This competence state was also lost rapidly when *Acinetobacter* spp. competent cells prepared in vitro were subsequently inoculated in oligotrophic soils (Nielsen et al. 1997). Several approaches for overcoming these limitations have been studied. Nutrient amendment and a high phosphate level led to *Acinetobacter* sp. BD413 competence in soil. In addition, transformation frequencies were also found to be higher in a silt loam than in a loamy sandy soil (Nielsen et al. 1997).

Nielsen and Van Elsas (2001) studied *Acinetobacter* sp. BD413 bacterial competence stimulation in sterile and non-sterile soil after addition of various organic compounds that are found naturally in the rhizosphere of crop plants. Mixtures of organic and amino acids such as acetate, lactate and alanine, sugars, mainly glucose, and the high-P salts have a pronounced effect on natural transformation frequencies of *Acinetobacter* sp. BD413 in non-sterile soil. Many compounds exuded into the rhizosphere by agricultural plants are able to increase bacterial metabolic activity (Kroer et al. 1998) and stimulate competence development and natural transformation (Nielsen and Van Elsas 2001).

Heterogeneity of the soil matrix must be seriously considered before concluding that soil environments are unable to provide favourable conditions for natural competence development. Bacteria can develop in biofilms on roots, decaying organic matter, and even mineral particles, thus improving the adapted conditions for DNA-transfer events including natural transformation (Nielsen et al. 1998; Paul 1999). Spatial distribution of bacteria at the microhabitat scale level is currently under investigation with specific and sensitive molecular methods, which should provide new data about the probabilities of contact between bacteria and extracellular DNA or other bacteria (Ladd et al. 1996; Grundmann and Debouzie 2000; Dechesne et al. 2003; Vogel et al. 2003).

15.6 Gene Transfer in the Environment by Alternate Genetic Transformation-Related Mechanisms?

One isolate belonging to the genus *Pseudomonas fluorescens* could not be naturally transformed in vitro under a wide range of conditions, but provided transformants when inoculated in sterile or non-sterile soil in the presence of plasmid DNA (Demanèche et al. 2001c). Similar results were obtained with *E. coli* that formed transformants in river water (Baur et al. 1996), foodstuff (Bauer et al. 1999), urine (Woegerbauer et al. 2002) and on agar plates (Tsen et al. 2002). These results indicate that in situ biotic and abiotic conditions regulate DNA uptake in a wider range of bacteria than those already referenced as being naturally transformable. Whether these bacteria undergo an active DNA-uptake process similar to that related to competence development or are subjected instead to a passive DNA entry due to chemically or physically mediated cell wall and membrane permeabilisation is still unclear. Other environmental conditions, such as those related to lightning discharges, can lead to the natural electro-transformation of bacteria in soil (Demanèche et al. 2001a; Cérémonie et al. 2004). Lightning-related electrical parameters, including current injection and electrical fields, are the same (when considered at the same spatial scale) as those delivered by electroporators that are used to transform a wide range of bacteria in the laboratory (Demanèche et al. 2001a).

15.7 Persistence of Extracellular DNA in Soil

A key factor of bacterial transformation frequency in soil is the availability of non-degraded and biologically active DNA after its release from dead (or living) cells. Availability means that extracellular DNA has to escape chemical and enzymatic degradation until a contact is established with a transformable bacterium. Most of the initial work that aimed at studying the occurrence of natural transformation-mediated gene transfer in soil focused on the detection of extracellular DNA in soil, and, thus, led to the study of DNA-degradation kinetics and mechanisms.

Significant amounts of non-degraded DNA were detected in natural soils (Frostegård et al. 1999). This confirmed results of microcosm-based experiments in which purified genomic (chromosomal or plasmidic) DNA from bacteria (Romanowski et al. 1992; Widmer et al. 1996; Gebhard and Smalla 1999), bacterial lysates (Nielsen et al. 2000a) but also plant DNA, ground plant material (Widmer et al. 1996), plant leaves (Widmer et al.

1997), decaying plant material (Ceccherini et al. 2003) and pollen (Meier and Wackernagel 2003b) were inoculated into soil before re-extraction.

Persistence of most extracellular DNA in soils was due to a reversible adsorption onto soil components including sand particles, humic compounds and particularly clay minerals, such as montmorillonite, illite, and kaolinite (Paget et al. 1992; Crecchio and Stotzky 1998; Demanèche et al. 2001b; see Chap. 7). The efficiency of the DNA-adsorption process depended on the size of the DNA molecules and their linear, open circular or super coiled conformations (Gallori et al. 1994; Poly et al. 2000; Demanèche et al. 2001b). Adsorption efficiency was also affected by soil parameters such as pH, temperature, concentration and cation valency (Romanowski et al. 1991; Khanna and Stotzky 1992; Lorenz and Wackernagel 1994). In addition to a specific protective effect of DNA adsorption, nucleases are also adsorbed onto clay particles leading to a physical separation between enzymes and their DNA substrate, thus increasing the protective effect of adsorption (Demanèche et al. 2001b). Finally, interactions between cell debris, including membranes and cytoplasmic residues, and DNA contributed to the prevention of rapid biological inactivation of DNA (Nielsen et al. 2000a).

Due to the protective effect of adsorption, the turnover of a significant part of extracellular DNA released into soil by various organisms including animals, plants, fungi or bacteria would be quite slow; this slow rate explains the positive detection by polymerase chain reaction (PCR) of DNA after its incubation for months and even years in soil (Paget et al. 1992; Romanowski et al. 1993; Widmer et al. 1996, 1997; Gebhard and Smalla 1999). However, hybridisation or PCR-based detection of DNA did not provide any information about the biological potential (transforming ability) that the persisting DNA could maintain throughout its incubation. Preliminary results based on inoculation of pure DNA in soil indicate that biological activity of DNA for competent bacteria decreased rapidly, in only a few hours (Gebhard and Smalla 1999), demonstrating a discrepancy between the physical stability and the functional significance of chromosomal DNA in soil (Nielsen et al. 2000a).

15.8 Development of Methods To Investigate Gene Transfer

Most of the in situ transformation experiments conducted recently have involved inoculation into sterile or non-sterile soil samples of donor DNA (plasmid or chromosome) in combination with bacteria already in a competent state. Broad host range plasmids were used allowing the transforming DNA to be replicated in various bacteria (Lorenz and Wackernagel 1994;

Timms-Wilson et al. 2002). When chromosomal DNA from bacteria or plants was used, specific constructions were developed in which one or two easily selective marker genes were flanked by homologous sequences in order to promote homologous recombination-mediated integration in the recipient host (de Vries et al. 2001). Detection of transformants required expression of the newly acquired marker genes to confer antibiotic resistance allowing the recombinant clones to grow under selective pressure. PCR and/or DNA hybridisations were used to confirm the presence of donor-specific DNA sequences in the recombinant clones. A derivative of this approach, the marker rescue system, was proposed in which donor and recipient DNA contained the same marker gene with the only exception that expression was inactivated in the recipient strain by deletion of the central part of the gene. Expression was rescued after transformation with donor DNA and homologous recombination between donor and recipient DNA based on the remaining sequences of the marker gene (de Vries and Wackernagel 1998; Gebhard and Smalla 1998; Nielsen et al. 2000b; Kay et al. 2002b; de Vries et al. 2003; Meier and Wackernagel 2003b). Moreover, the deleted region was used as a probe to demonstrate that the rescued phenotype was due to the expected transfer event. The rescue marker systems were used to study the potential of antibiotic resistance genes from genetically modified plants to be transferred to bacteria. The system was adapted with the *nptII* gene used in nuclear-modified potatoes and sugar beet plants and the *aadA* gene from transplastomic (the chloroplast genome was modified) tobacco plants that were cloned after deletion in *Acinetobacter* spp. and/or *Pseudomonas stutzeri* (Nielsen et al. 2000b; Kay et al. 2002; de Vries et al. 2003). Transformation with plant DNA restored a complete and functional *nptII* or *aadA* gene conferring resistance to kanamycin and spectinomycin-streptomycin, respectively. Applications of these specific and sensitive approaches included determination of the transformation potential of plant DNA which persisted in soil. In most cases, total DNA was extracted from soil and used to transform the recipient bacteria in vitro (Timms-Wilson et al. 2002; Meier and Wackernagel 2003b). Another application deals with the use of the recipient strain in situ (soil or plant) which requires the subsequent and specific isolation of transformants. This approach remains problematic in soil environments which contain a significant proportion of antibiotic-resistant indigenous bacteria.

If the detection of transformation-mediated gene-transfer events that would have occurred naturally in situ among indigenous soil bacteria remains extremely difficult due to a lack of effective marker sequences, there are some possibilities for detecting bacteria that would have acquired the genes from transgenic plants. In numerous cases, these plants contain marker genes, such as antibiotic-resistance genes, which could facilitate the selection of bacteria which would have acquired these genes. This approach

requires, however, selecting between the recently generated transformants and indigenous bacteria naturally fitted with the same gene. This can be done by detecting sequences specific to the plant construction that flank the marker gene and that could have been co-transferred to bacteria. Presence of these transgenic plant-specific synthetic DNA sequences in soil bacteria could demonstrate that plant-bacteria DNA transfer had occurred. However, to date there is no report of the isolation of bacteria in which plant DNA sequences can be detected from soils cultivated with transgenic plants.

15.9
Gene Transfer by Natural Transformation from Transgenic Plants to Bacteria – A Possible Event?

Plant transgenes may behave like any other naturally occurring genes in the soil environment but they often differ in several ways from native genes with respect to their likelihood of gene transfer, expression in the new host, and selection. A successful (and detectable) gene transfer from transgenic plants to bacteria requires several events to occur successively, thus justifying that each of the steps was investigated separately to determine the limiting factor. During the first step, DNA must be released by the plant. A study by Ceccherini et al. (2003) demonstrated that most of the DNA was degraded during the plant decaying process. Only a minor fraction of the plant DNA was susceptible to a release into the soil. These data indicate that endophyte bacteria, including pathogens and other colonising bacteria, are those for which exposure to plant DNA would be the most significant (Kay et al. 2002a). In addition to a favoured access to plant DNA, plant tissues could be environments in which bacteria easily reach competence. However, the main barrier to gene transfer would not be a physical one (degradation and/or unavailability of the DNA) or a physiological one (inability of bacteria to take up DNA), but a genetic barrier that prevents exogenous DNA being integrated in the recipient genome of bacteria. The results of Kay et al. (2002b), which demonstrate that a marker gene from a transplastomic plant could be transferred in planta only to bacteria possessing the appropriate homologous DNA sequences, confirm the fundamental role of the recombination step in regulating gene transfer. However, transfer of plant transgenes with a low level of homology with bacteria genomes cannot be excluded considering that short homology sequences can act as anchor sequences to promote illegitimate recombination of longer DNA fragments (de Vries et al. 2004). Although these events occur at low frequency, they confirm that the exchange of DNA between distantly related organisms including plant and bacteria is theoretically possible.

15.10 Concluding Remarks

Environmental microbiologists have developed numerous studies with the objectives of providing direct and experimental evidence of the impact of horizontal gene transfer (HGT) events on bacterial genome structure. In silico analysis of complete genome sequences has led to proposals of relatively high HGT rates. However, in spite of these efforts, there is still a discrepancy between the two approaches, and additional experimental studies are necessary to confirm the actual role of these mechanisms in complex environments such as soil or sediment. Location, density, and metabolism of bacteria in soil microenvironments are still almost unexplored, and their ability to develop competence is unknown. For example, if plant DNA was detectable in soils for years (Paget et al. 1992; Gebhard and Smalla 1999), there was no clear evidence that this DNA could be still involved in a transformation process with bacteria. However, two points must be considered carefully when addressing HGT by natural transformation in soil environments. The first is that studying genetic information transfer between indigenous bacteria remains very difficult because of the lack of marker genes that could allow differentiation between recipient and recombinant bacteria. Most studies that provided the available data involved inoculation of donor or recipient bacteria but such approaches are known to simulate poorly what might occur with indigenous bacteria which do not colonise the same soil microniches as inoculated bacteria. This is why transgenic plants with marker genes inserted in their genomes have to be considered as invaluable and perhaps the only model for studying how genetic information can be exchanged naturally between living organisms.

The second point is that experimental studies were conducted at the macroscale level without considering the spatial distribution of bacteria at the microscale level which is the level at which bacteria interact in soil. Tools need to be adapted to gene-transfer potentials in the few spots colonised by bacteria considering that 10^8 cells per g of soil occupy only 0.1 mm^3 of the 500 mm^3 of pores in 1 cm^3 of soil (Pallud et al. 2004).

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16 Reporter Genes in Bacterial Inoculants Can Monitor Life Conditions and Functions in Soil

Jan Sørensen, Ole Nybroe

16.1 Introduction to Reporter Bacteria

Application of reporter bacteria in complex environmental systems is a great challenge to environmental microbiologists and the technology continues to develop at high speed. The specific aim of this chapter is to provide an overview of recent reporter applications in soil environmental systems. Previously, Sørensen (1997) and Sørensen et al. (2001) reviewed some of the advances obtained by marker and reporter gene techniques in soil systems, discussing the distribution and activity of pseudomonads in soil and rhizosphere habitats. Since then, many new and fascinating observations have been made, as will hopefully be clear from the following sections, which will focus on studies using reporters for metabolic activity, nutrient and oxygen availabilities and for aromatic pollutants.

The present review does not intend to cover all aspects of the new reporter methodology in environmental microbiology (for recent reviews, see e.g. Köhler et al. 2000; Hansen and Sørensen 2001; Keane et al. 2002; Leveau and Lindow 2002; Jansson 2003), nor does the review cover a detailed treatment of the molecular tools for reporter construction or details of sensing or signal transmission, gene regulation, green fluorescent protein (GFP) chemistry, or optical detection systems (Tsien 1998; Leveau and Lindow 2001; Van der Meer et al. 2004). Chapter 17 in this volume, entitled "Reporter gene technology in soil ecology: detection of bioavailability and microbial interactions", presents a detailed description of different types of reporter bacteria and the properties of the most common reporter genes. Furthermore, methodological considerations concerning assay format and the concept of bioavailability are treated in more detail in that chapter. In this brief introduction we will, however, highlight essential definitions and mention the most important advantages and limitations associated with the general use of reporter bacteria for environmental applications.

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Reporter bacteria, also referred to as whole-cell biosensors, bacterial bioreporters or monitor strains, are equipped with reporter genes that encode a product, which is easily assayed and related to metabolic activity or specific gene expression of the host cell. For application in soil, the reporter genes encoding bioluminescence (the bacterial or firefly luciferase genes, *lux* or *luc*) and fluorescence (genes for expression of the green fluorescent protein *gfp* and derivatives thereof or *DsRed*) are more useful than conventional reporter genes such as *lacZ* (encoding beta-galactosidase) or *xylE* (catechol 2,3-dioxygenase) due to higher sensitivity and specificity as well as the option for non-destructive real-time analyses in situ.

Reporter bacteria can be classified into three overall groups. The first group is the non-specific reporters, which carry *lux* reporter genes under control of a constitutive promoter (also called marker genes). Frequently, these reporters are tagged with a complete *luxCDABE* cassette encoding the luciferase as well as genes involved in production of an aldehyde substrate. These bacteria will emit constant light when supplied with oxygen and energy. As such a *lux*-tagged reporter is dependent on the intracellular energy from oxic respiration, reduced bioluminescence is a sensitive, but non-specific assay format for testing environmental stress conditions, e.g. toxicity of soil pollutants. The principle of a non-specific reporter strain is illustrated in Fig. 16.1, but it should be noted that non-specific reporter constructs can be plasmid-borne as well as chromosomal. Non-specific reporter strains with constitutive expression of both *gfp* and *lux* under the same promoter may offer simultaneous detection of cell localisation and metabolic activity in soil (Unge et al. 1999). The second group of reporters is based on the expression of reporter genes in response to generally stressful stimuli (semi-specific reporters). Finally, the third group of reporters responds to a very specific stimulus (specific reporters). Specific reporters may respond to the presence of specific compounds or elements (e.g. Jaeger et al. 1999) or to their absence (e.g. Koch et al. 2001). Figure 16.2 illustrates the general principle of a specific reporter.

Reporter bacteria respond to compounds or elements in their surroundings that are biologically available to the host organism. Hence the bioavailable fraction of a compound is defined through the ability of this fraction to change gene expression in the reporter strain. It is of fundamental importance that the amount of bioavailable compounds, defined as above, can only be assessed in relation to the specific reporter strain. Other microorganisms may have alternate mechanisms regulating their gene expression in response to the compound in question, or have different permeability properties than the reporter strain. This is a fundamental limitation of the technique as we cannot be absolutely sure that assessments made by using a specific reporter strain are valid for indigenous microorganisms, even for taxonomic groups closely related to the reporter strain.

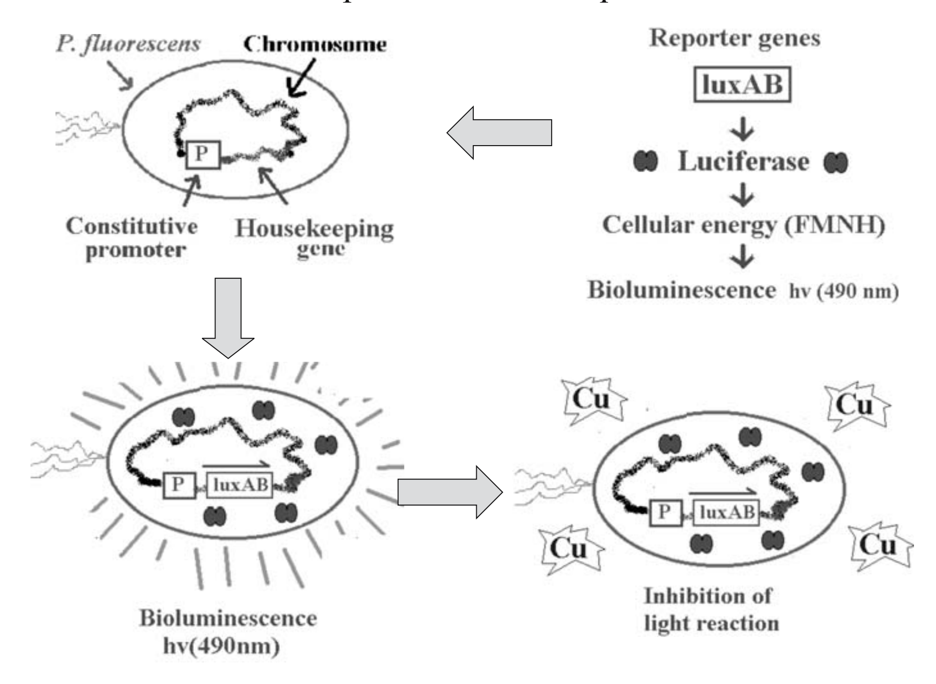


Fig. 16.1. Principle of a non-specific bacterial reporter. The *luxAB* genes encoding bacterial luciferase are inserted behind a constitutive promoter located on the chromosome of *Pseudomonas fluorescens*. Bioluminescence depends on cellular energy, but also on oxygen and aldehyde substrate (not shown). In non-specific reporters carrying the entire *luxCDABE* operon, the aldehyde substrate is produced by proteins encoded by *luxC*, *luxD* and *luxE*. Toxic compounds or elements such as Cu inhibit generation of cellular energy and thereby the light reaction

The terms bioavailable and bioavailability are often used in a somewhat unprecise way. The bioavailable fraction can be defined as stated above, and a tight definition of bioavailability would be as follows: the fraction of the total pool of a compound that is biologically available to the reporter strain. Consequently, to address bioavailability of a compound, it is necessary to perform concomitant chemical and biological analyses. In this respect the sensitivity of chemical assays versus whole-cell biosensors becomes an important issue. As discussed recently by Van der Meer et al. (2004), chemical methods typically detect polluting compounds with a sensitivity in the order of ng l^{-1} whereas the sensitivity of assays based on reporter bacteria typically is lower.

The chemical methods rely on extraction and further processing of the samples. Several reporter applications in soil can be made in parallel to

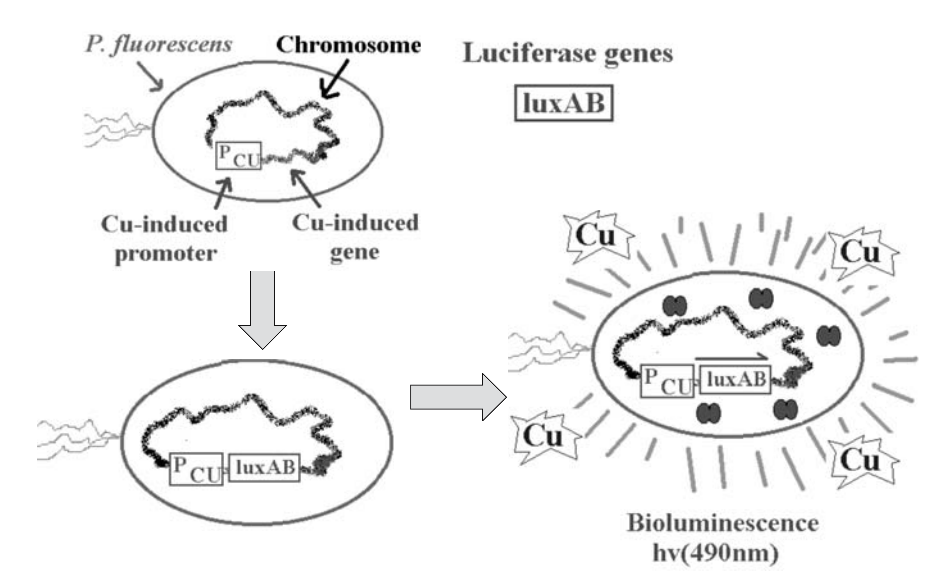


Fig. 16.2. Principle of a specific bacterial reporter. The *luxAB* genes encoding the bacterial luciferase are inserted behind a Cu-induced promoter located on the chromosome of *Pseudomonas fluorescens*. Expression of bioluminescence occurs in the presence of Cu in concentrations below the level that inhibits the light reaction, see legend to Fig. 16.1

such chemical analyses as they are made for analysis of extracts or slurries. The advantage is consequently that biological and chemical analyses can be compared and bioavailability of a given compound or element can be determined. However, with this set-up the reporter assay merely provides an indirect or semi-quantitative estimate of in vivo conditions. For bioluminescent and fluorescent reporters, which have been introduced into the soil environment, it is possible to perform non-destructive in vivo analyses. For these applications a meaningful comparison of chemical and biological data is difficult to perform, and the introduction of a high number of reporter cells into a soil environment may also affect the results. Hence, the introduced cells may adsorb the compound in question onto their surfaces, thereby affecting bioavailability. Furthermore, the distribution of a reporter inoculum in the soil matrix is probably not comparable to that of the indigenous population during the brief time period during which the introduced cells are able to emit a diagnostic signal, see e.g. Højberg et al. (1999). As a consequence we should be cautious when interpreting results obtained by so-called in vivo reporter assays, since the reporter cells might still not experience the realistic physical and chemical conditions in the soil. Future development of methods and assay concepts

needs to demonstrate with confidence that reporter genes in bacterial inoculants provide a realistic picture of life conditions and functions in the soil.

16.2 Applications of Reporter Bacteria in Soil

16.2.1 Non-specific Reporters of Metabolic Activity

The non-specific reporters of metabolic activity were used relatively early to monitor metabolic activity of bacterial inoculants in samples from soil and rhizosphere. As mentioned above, for a *lux* reporter gene cassette under control of a constitutive promoter, the bioluminescence signal is solely dependent on cellular activity provided that oxygen and the luciferase aldehyde substrate are available in surplus. Using this reporter system in *Pseudomonas fluorescens* strains introduced into soil, Meikle et al. (1995) reported a loss of metabolic activity over time in drying soils. With a comparable approach, Kragelund et al. (1997) found that metabolic activity in a root-colonising *P. fluorescens* strain varied along the root and was different on the root surface (rhizoplane) compared to the surrounding rhizosphere and bulk soil. Finally, the dual *gfp*-*lux* system in *P. fluorescens* constructed by Unge et al. (1999) presented detailed images of both cellular localisation (*gfp*) and metabolic activity (*lux*) of the reporter strain after inoculation in soil.

For environmental toxicity testing, bacterial reporters of metabolic activity were soon commercialised by the Microtox assay, where acute sample toxicity is measured by light reduction of reconstituted freeze-dried *Photobacterium phosphoreum* (Bulich and Isenberg 1981). To address the criticism that reporter bacteria should be relevant in soil testing, *lux*-modified constructs of several soil isolates, e.g. *P. fluorescens* and *P. putida*, *Rhizobium* spp. and *Burkholderia* spp., have been made to assess the acute toxicity of heavy metal and xenobiotic contaminants in groundwater and soil samples. One early work was based on a *P. fluorescens* strain (10586s) equipped with a complete *luxCDABE* cassette on the multi-copy plasmid pUCD607; the reporter strain showed toxicity stress when exposed to xenobiotic pollutants such as chlorobenzenes in the groundwater samples (Boyd et al. 1997). Using the *P. fluorescens* 10586s reporter for toxicity testing of chlorophenols, the work by Palmer et al. (1998) illustrates another application; acute toxicity to penta-chlorophenol (PCP) arising from deposition of paper mill sludge on the field soil was demonstrated in soil extracts. Many variants of tri-, di- and monochlorophenols (TCP, DCP and CP) occur in

soil and it has been of interest to compare structure-toxicity relationships and responses in different reporter bacteria. Boyd et al. (2001) reported that small differences between acute toxicity responses towards several chlorophenols in the *P. fluorescens* 10586s *lux* reporter and a *Burkholderia* sp. (Rasc strain) with the same plasmid (pUCD607) were probably due to structural differences in the bacterial membrane. However, both strains still demonstrated the typical correlation between a high degree of chlorination/hydrophobicity and increasing toxicity. Finally, Weitz et al. (2001) compared the acute toxicity responses in two *Pseudomonas* strains (*P. fluorescens* 8866 and *P. putida* F1) equipped with chromosomally integrated *luxCDABE* (controlled by a strong, undefined promoter) and found quite comparable responses to a chlorophenol (3,5-DCP) and two heavy metals (Zn and Cu).

Surfactants (detergents) are important constituents of municipal sludge deposited on agricultural soil; as for the phenolics mentioned above, significant cytotoxicity of the surfactants may be due to their damaging of target cell membranes. A recent application of reporters to test surfactant toxicity in soil by Brandt et al. (2002) used a metabolic *luxAB* reporter in *Nitrosomonas europaea*; in soils amended with LAS (linear alkylbenzene sulphonate) surfactant, the authors found similar EC_{50} values (approx. 50 mg kg^{-1}) in two different soils. The relatively high EC_{50} values and a rapid degradation in the field soils suggested that LAS disposal through sludge was not a major environmental problem. Where controlled application of surfactant is actually desirable, e.g. to solubilise organic pollutants in bioremediation, both toxicity and degradation of the pollutants may be followed by detergent-resistant reporters. Layton et al. (1999) presented such surfactant-resistant soil bacteria (*Stenotrophomonas* and *Ralstonia*) marked with *lux* (metabolic activity), intended for toxicity testing in soil undergoing bioremediation.

16.2.2 Semi-specific Reporters of Stress

Since bacterial physiology under many adverse conditions (e.g. exposure to high temperature or osmolarity, drought, or presence of reactive oxygen species) is controlled by global regulatory circuits (regulons) of gene expression, reporter constructs based on such circuits may be used as "general" or "semi-specific" stress reporters. Typically, these reporters will respond to a broad range of conditions and can be useful to characterise a soil environment in terms of general cytotoxicity. Due to the extended knowledge on such systems in enteric bacteria, *Escherichia coli* or *Salmonella typhimurium* was used in much of the early work, e.g. linking

the *lux* reporter system to "general stress" promoters such as the heat shock promoters *dnaK* or *grpE* (Van Dyk et al. 1995). A recent contribution by Park et al. (2002) demonstrated the use of a *dnaK-luxCDABE* construct in a *Pseudomonas* sp. (strain DJ-12) to detect aromatic compounds (biphenyl and 4-chlorobiphenyl) related to PCB occurrence and degradation in the environment (see also below). A panel of bioluminescence-based stress reporters for environmental toxicity testing has been constructed in *E. coli* based on expression of protein-damage sensitive (*grpE*), oxidative-damage sensitive (*katG*) and membrane-damage sensitive (*fabA*) promoters (Van Dyk et al. 1995; Belkin et al. 1996, 1997). The potential of these reporters may be illustrated by the recent work of Bechor et al. (2002) who used the *E. coli* reporter with *fabA-lux* to show a particularly high toxicity (membrane damage) of detergents (SDS, Tween and Triton variants) compared to phenolics and BTEX (e.g. benzene, toluene and xylene) compounds (see also below). Undoubtedly, several of these reporter systems may also be constructed in indigenous soil bacteria, e.g. *Pseudomonas* spp.

Representing another global regulon, the DNA damage-inducible promoters of the *recA* and *colD* genes have been linked with *lux* in *E. coli* or *S. typhimurium* (Van Dyk et al. 1994; Vollmer et al. 1997; Rosen et al. 2000; Rabbow et al. 2002); such reporters based on the SOS response involving DNA repair have been proposed as microbial indicators (SOS-LUX test) of genotoxicity in environmental samples (Rabbow et al. 2002). Representing a non-enteric reporter for such environmental application, the fusion of *lux* genes to the *recA* promoter of *P. aeruginosa* was used to monitor UV effects on natural bacterial populations (Elasri and Miller 1998).

16.2.3 Reporters of Bacterial Growth (Ribosome Synthesis)

A few specific reporters of bacterial growth have been constructed in *Pseudomonas* bacteria by fusing bioluminescence or *gfp* reporter genes to the promoter regions of operons supporting synthesis of ribosomal RNA. An early example is the work of Marschner and Crowley (1996) who used a ribosomal promoter-driven *lux* reporter (emitting bioluminescence during growth, when rRNA genes are highly expressed); the authors reported that growth of a *P. fluorescens* strain was higher in natural rhizosphere (pepper) than in bulk soil. Ramos et al. (2000), using a ribosomal promoter-driven *gfp* reporter (unstable GFP variant) in a *P. putida* strain, found that bacterial growth was detectable in rhizosphere (barley), primarily at the root tip.

16.2.4 Reporters of Nutrient Limitation

Responses to specific nutrient limitation in soil and rhizosphere represent some of the first applications of reporter bacteria to study specific gene expression in this environment. The field has been dominated by intensive studies of strains of *Pseudomonas* spp., most certainly due the importance in degradation and nutrient cycling by this organism per se, but also due to the potential exploitation of the organism in agricultural biotechnology, e.g. for plant protection, plant-growth promotion and bioremediation. In the following the focus is placed on the major results obtained for reporter studies on the soil C, N and P limitations, presence of specific C or N sources and on molecular oxygen availability.

Carbon Limitation

Root exudates have long been considered to be the major C source supporting growth of root-colonising bacteria in the rhizosphere of young plants. A recent advancement has been the use of non-specific *lux*-tagged reporters to detect the actual C-source composition and availability in soil and rhizosphere samples through changes in metabolic activity. Inoculant *Pseudomonas* spp. (shortly pre-starved for C) responded to both source and concentration of C; wheat root exudates gave a response comparable to that of a reducing sugar monomer (glucose), rather than that of common amino acid (glutamate) or carboxylic acid (succinate) components in root exudate (Yeomans et al. 1999). The cells were later shown to be capable of discriminating the composition of root exudates from plants grown with or without herbicide treatment (Porteous et al. 2000). Koch et al. (2001) and Van Overbeek et al. (1997) both used specific *lacZ*-based C-limitation reporter systems in *P. fluorescens* strains to demonstrate C limitation in bulk soil, but not in rhizosphere. While in general C may not be limiting in the rhizosphere, the composition and availability of specific organic components can still be important for actual C status of *Pseudomonas*.

Nitrogen and Phosphorus Limitation

The significance of N and P limitation in soil and rhizosphere has also been addressed by studies including bioluminescent *Pseudomonas* reporter strains (Kragelund et al. 1997; Jensen and Nybroe 1999; Koch et al. 2001; Standing et al. 2003). In bulk soil neither N nor P limitation could be observed in agreement with the above observations of C limitation in this habitat (Kragelund et al. 1997; Jensen and Nybroe 1999). However, soil amendment with barley straw changed the life conditions for the inoculated *Pseudomonas* strain, which encountered N limitation as C-rich polymers

from the barley residues were degraded (Jensen and Nybroe 1999; Koch et al. 2001). The rhizosphere (barley) demonstrated significant N limitation, whereas P limitation was not observed (Kragelund et al. 1997; Jensen and Nybroe 1999). This work was the first identification of a major nutrient limitation by N of potential significance for growth and activity of pseudomonads in natural rhizosphere. An important recent development is the double reporters, which e.g. can address changes in nutrient availabilities in one reporter strain (Koch et al. 2001). Even the concomitant application of several reporters addressing C, N and P availabilities in the same samples (Standing et al. 2003) will probably be useful for future dissections of the nutrient conditions significant to growth and survival of *Pseudomonas* spp. in soil environments.

16.2.5 Reporters for Specific Carbon and Nitrogen Sources

The N reporter strain used in the work cited above reacted towards limitation by both NH_4^+ and common amino acids (e.g. glutamate), and further work should address if specific N components in exudates may regulate growth of *Pseudomonas* spp. in the rhizosphere. Specific reporter bacteria responding to individual amino acids show great promise for identification of such growth-limiting compounds. For example, induction of a lysine-responsive *P. putida* reporter was demonstrated in rhizosphere (corn), but not in bulk soil (Espinosa-Urgel and Ramos 2001).

The study by Jaeger et al. (1999) illustrates another advantage of reporter studies, namely that precise information on the spatial distribution of C and N compounds can be obtained. The employed *Erwinia herbicola* tryptophan-reporter strain carried a fusion between the *aatl* gene encoding a tryptophan aminotransferase and the *inaZ* ice nucleation marker. The strain was introduced into the rhizosphere of an annual grass (*Avena barbata*), extracted, and ice nucleation activity was measured in samples from bulk soil and in samples taken at different sections of the roots. The reporter showed significant induction in older root segments with lateral root formation but not at the root tip (Jaeger et al. 1999). In contrast, bioavailable sucrose was most abundant at the root tip as demonstrated by another *E. herbicola* reporter strain used in the same set-up. Finally, a *P. fluorescens* reporter strain was recently used by Kuiper et al. (2001) to show that uptake regulation of putrescine, a common polyamine in root exudate (tomato), was important for growth rate and thus competitive colonisation ability in the rhizosphere.

16.2.6
Reporters for Oxygen Limitation (Anaerobiosis)

Oxygen availability in the soil environment is of fundamental importance to expression of several distinguishing traits in *Pseudomonas* spp., notably denitrification, but also a number of redox-regulated traits like fluorescent siderophore and HCN production. High consumption and limited supply rates of oxygen may be expected in rhizosphere, organic aggregates (hot spots), or highly compacted soil. In the first attempt to determine oxygen availability by reporter strains, Højberg et al. (1999) found induction of a low-oxygen-sensitive *lacZ*-based *P. fluorescens* reporter strain in wetted (85% WHC) but not in unwetted (60% WHC) rhizosphere (barley) and in compacted bulk soil. The work demonstrated that common water and texture conditions easily promoted low-oxygen and thus denitrifying conditions in both rhizosphere and bulk soil. More work based on reporter strains or on growth potential of mutant strains is needed to elucidate the role of redox-regulated phenotypes in soil; the latter approach was used by Ghiglione et al. (2000), using a nitrate reductase-deficient mutant of a denitrifying *P. fluorescens* strain and demonstrating that this function may confer a selective advantage in the rhizosphere (corn).

16.2.7
Reporters of Aromatics and Their Degradation

Reporter bacteria of organic pollutants have been constructed in several, relevant *Pseudomonas* and *Ralstonia* strains and are useful to determine occurrence and degradation of these compounds in the soil environment. The discovery of substrate-specific transcriptional activators (regulator proteins) has made possible the development of specific reporters of several aromatic groups. The reporters can be engineered when the reporter gene is under control of a transcriptional activator, e.g. naphthalene-responsive NahR, toluene-xylene-responsive XylR, benzoate-responsive XylS or phenol-responsive DmpR, sensing the aromatic or aromatic group in the environment. Under suitable conditions, the increase in gene product such as bioluminescence using *lux* or *luc* reporters is then correlated with the organic contaminant concentration. Köhler et al. (2000) provided an overview of some of the recent reporters, including their approximate limits and concentration ranges of detection. The *lux*- or *luc*-based data derived from both *E. coli*, *P. fluorescens*-*P. putida* or *Ralstonia* spp. reporters indicated suitable concentration ranges of 100 nM to 100 μM for commonly attended, aromatic pollutants such as naphthalene, toluene-xylene and PCB. In the following, further treatment is given to these compounds and some of the bacterial reporters detecting their occurrence and degradation in soil.

Naphthalene and the PAH Group

An early example (Burlage et al. 1990) is the naphthalene-degrader *P. putida* RB1353 (or RB1351) harbouring the catabolic NAH7 plasmid. The strain also contains a constructed pUTK9 reporter plasmid with a fusion of *luxCDABE* to the *nah* genes representing the "upper pathway" leading from naphthalene to salicylate controlled by a salicylate-inducible promoter (P*nah*). Another early naphthalene degradation reporter (King et al. 1990) is the *P. fluorescens* HK44 harbouring the same NAH7 catabolic plasmid but with a fusion of *luxCDABE* to the *nahG* gene representing the "lower pathway" leading from salicylate and on, also controlled by a salicylate-inducible promoter (P*sal*). Both P*nah* and P*sal* are recognised by the salicylate-activated NahR regulator and transcription is therefore dependent on salicylate concentration. Both reporters provide a quantitative measure of the degradation of naphthalene via formation of the obligate intermediate salicylate. Heitzer et al. (1992) used the *P. fluorescens* HK44 *nahG-lux* reporter in soil extracts and slurries; detection in the nanomolar range was established at concentrations over two orders of magnitude.

Since the *nahG-lux* reporter is metabolite-controlled (salicylate), the possibility is raised that it may be used as a semi-specific sensor for biotransformation of a whole group of PAH compounds. In addition to the two-ringed naphthalene compound, the *nah* operon is known to mediate degradation of the three-ringed phenanthrene and anthracene (Sanseverino et al. 1993). Finally, certain substituted naphthalenes, containing electrically neutral substituents on one of the rings, may also be fully degradaded via the salicylate intermediate in *P. fluorescens* (LeBlond et al. 2000) and may thus lead to bioluminescence in a *nahG-lux* reporter.

Benzene and the BTEX Group

The use of reporter bacteria to study degradation of the BTEX group (benzene, toluene, xylene) of aromatic solvents also serves to illustrate that reporter methodology must often deal with complex gene regulation. Analogous to the naphthalene degradation pathway, the well-studied toluene degradation plasmid (TOL, pWW0) of *P. putida* contains: (1) the "upper pathway" operon (Pu promoter and XylR regulator protein) coding for toluene and *m*- and *p*-xylene degradation to benzoate and toluates, respectively; (2) the "lower pathway" operon (Pm promoter and XylS regulator protein) coding for the subsequent degradation of benzoate (and toluates) to catechol and further (by meta-type ring cleavage) to tricarboxylic acid (TCA) cycle intermediates. Reporter systems can be made based on the inducible promoters of these degradation genes. However, it is most important to consider the specificity of the transcriptional activator proteins, XylR and XylS, since several compounds may act as inducer signals.

Since BTEX compounds are important environmental pollutants, attention has already been paid to construct bacterial reporters which may be used as inoculants indicating in situ degradation activity. An early example of a *luxCDABE*-based reporter to monitor degradation of BTEX compounds in the soil environment is the TOL plasmid-containing *P. putida* RB1401 (Burlage et al. 1994); this reporter responded to both toluene and xylene (*o*-, *m*- and *p*-isomers), indicating bioavailable pools of these compounds in the tested soil. Willardson et al. (1998) constructed a BTEX reporter in *E. coli* which contained the *luc* reporter gene under control of the Pu promoter and XylR regulator protein; this reporter detected toluene at low concentration (approx. 30 mg kg^{-1}) in soil.

Another, recent example is the *P. fluorescens* A506pTS reporter (Stiner and Halvorsen 2002) which has a fusion between *gfp* and the *tbuA1* gene promoter, from which toluene-benzene-utilisation genes (toluene monooxygenase and others) are transcribed. A variant, *P. fluorescens* A506p TolLHB, further extended the sensitivity towards toluene into the nanomolar range (Casavant et al. 2003); the stimulated light response by reporter cells in soil microcosms (barley roots) indicated that natural inducers of the *tbuA1* promoter were present in the non-polluted rhizosphere. Further, since a higher expression was localised in distinct regions along the roots, the reporter indicated a spatial heterogeneity of the plant- or microbe-derived release of inducing compounds. This work illustrated the need for detailed microscale studies of biodegradation activity in the soil-rhizosphere environment.

Phenol and Phenolics

The prototype is here the *Pseudomonas* sp. CF600 with the phenol degradation plasmid pVI150 (Shingler et al. 1993), containing the *dmp* genes (with Po promoter and DmpR regulator protein) for the catabolism of phenol and methyl-phenols (cresols) via hydroxylation and a subsequent meta-type cleavage pathway. A fusion between *luxAB* and the DmpR-responsive Po promoter was made in *Pseudomonas* sp. CF600 by Fernandez et al. (1994); a similar degradation reporter plasmid with a fusion between *luxAB* and a DmpR-responsive *dmp* gene promoter was made in *P. putida* KT2440 (pVI360; Shingler and Moore 1994).

While some biodegradation reporters respond to a single group or a narrow combination of organic pollutants, others have been devised to respond to a broader spectrum, e.g. when based on the “bottleneck” genes shared by many compounds for degradation and transport. *P. putida* TVA8 (Applegate et al. 1998) contains a chromosomal fusion of *luxCDABE* to the *tod* genes (toluene dioxygenase and others), controlled by the P*tod* promoter and thus providing a light response to not only benzene, toluene and xylene

(*m*- and *p*-isomers), but also to phenol. Further, new reporters responding to a broader substrate spectrum may also be constructed exploiting the cross-reactivity between structurally similar regulator proteins, e.g. the XylR regulator (toluene and xylene degradation), and the resembling DmpR regulator (phenol and cresol degradation). Finally, Wise and Kuske (2000) have demonstrated that DmpR mutants, representing small changes in the sensing part of the protein, may have altered substrate specificity of the regulator and thus of DmpR-controlled reporter expression. Hence, in addition to the prospect of discovering new beneficial strains with extended degradation repertoire, these observations may allow for design of new reporter bacteria to monitor a composite pattern of pollutant aromatics and their degradation in the environment.

Chlorophenols and Other Chlorinated Aromatics

Chlorinated aromatics represent a much-attended topic for environmental microbiologists, including use of reporter bacteria to assess bioavailability, toxicity and degradation rates. Metabolic toxicity reporters harbouring bioluminescence genes were mentioned above. Such reporters are particularly useful when degradation studies are conducted with highly toxic pollutants or their intermediates, e.g. phenols or chlorophenols (CP). The phenolic compounds are known to act as uncouplers of electron transport, thus leading to a reduced light output in bioluminescence-based reporters. A recent example was the use of *luc* reporter genes in 4-CP-degrading *Arthrobacter chlorophenolicus* in soil; maintenance of highly luciferase-active cells at high 4-CP levels in soil documented that the organism was well suited for cleaning-up (bioremediation) of the soil pollution (Elvang et al. 2001).

Recently, several specific reporters containing bioluminescence or *gfp* fusions in the functional degradation genes have been presented. A first example to illustrate the recent progress being made to detect occurrence and degradation of specific chlorinated aromatics involves the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and the degrading soil bacterium, *Ralstonial eutropha* JMP134. This strain, harbouring the degradation plasmid pJP4, has become the model of 2,4-D degradation; two modules of *tfd* genes, $R_{regulatory}$-$D_{II}C_{II}E_{II}F_{II}$ and $T_{regulatory}$-$C_{I}D_{I}E_{I}F_{I}$, are both involved in the degradation steps from ring cleavage of 2,4-dichlorocatechol to formation of a product entering the TCA cycle. Hay et al. (2000) constructed a reporter containing a chromosomal insert of the *tfdR-tfdD*$_{II}$ (including promoter) sequence linked to promoterless *lux*; the organism responded sensitively and linearly (up to 100 µM) to both 2,4-D and the first degradation intermediate 2,4-dichlorophenol (2,4-DCP). Early applications of the reporter in soil slurries showed a distinct but delayed response to aged 2,4-D residues; more studies are needed to understand the relationship between

this lag in bioluminescence and bioavailability of the herbicide residues in the soil. In a recent report, Füchslin et al. (2003) presented an alternative reporter containing a chromosomal insert of the *tfdC*$_I$ (including promoter) linked to *gfp*; the reporter system was supported by the indigenous regulator from *tfdT* located on pJP4. Such a reporter construct appears promising for application in soil environments, e.g. for single-cell studies using advanced microscopy.

A final example of pollutant compounds being assessed with reporter bacteria in soil degradation studies is that concerning the polychlorinated biphenyls (PCB). This group of much-attended pollutants is degraded by several soil bacteria, including typical degrading genera such as *Ralstonia*, *Burkholderia* and *Pseudomonas*. The initial degradation step is mediated by a biphenyl dioxygenase, encoded by the *bphA* gene, and Layton et al. (1998) constructed an *R. eutropha* ENV307 strain harbouring the reporter plasmid pUTK60 with an insert of *orf0*$_{\text{regulatory}}$-*bphA*$_1$ (including promoter) linked to promoterless *lux*. The detection limit was approx. 1 μM for both monochlorinated (2-CB, 3-CB and 4-CB) and polychlorinated biphenyls (Arachlor 1242 mixture), but the linear range appeared narrow (approx. 1–10 μM). By comparison, Brazil et al. (1995) constructed a PCB reporter in *P. fluorescens* F113 by chromosomal insertion of *orf0-bphA*$_1$ linked to promoterless *lacZ* (beta-galactosidase). This strain was a strong root coloniser showing potential for bioremediation of polluted soils; however, since the organism did not naturally contain biphenyl-degrading genes, a complete array of *bph* genes was inserted independently of the reporter genes. The authors demonstrated that the reporter strain had beta-galactosidase activity and thus expression of *bph* genes for at least 5 days after inoculation on sugar beet seeds. Recently, Boldt et al. (2004) modified the PCB-degrading *P. fluorescens* F113 strain to report on *bph* activity by expression of stable or unstable GFP (Andersen et al. 1998). Using confocal laser scanning microscopy (CLSM) these authors could discern single F113 cells showing *bph* activity (and thus biphenyl/PCB degradation) on roots developing from inoculated alfalfa seeds in PCB-amended soil extract medium.

16.3 Current and Future Trends

Given the current excitement of novel possibilities arising with the reporter technology, it is also timely to warn against the pitfalls sometimes neglected and the needs for testing and standardisation. Meeting such a standard, a thorough testing of *lux* reporter systems has recently been conducted by Maier and co-workers (Neilson et al. 1999; Dorn et al. 2003). With particular

attention to soil studies, reporters are used in their most difficult environment, extremely heterogeneous at the bacterial scale. Leveau and Lindow (2002) recently discussed the use of reporter bacteria in heterogeneous samples; it is clear that reporter bacteria actually offer a unique opportunity to study this heterogeneity in soil in great detail. In the future, more studies should be made to compare results using different scales of investigation; there are unique opportunities in comparing single-cell studies based on sensitive, fluorescent reporters such as *gfp* in combination with advanced CLSM with larger samples such as soil extracts or slurries, and with whole plant-soil detection studies based on direct bioluminescence recorded under a sensitive camera. On-line monitoring using immobilised reporter cells on optic fibres (Heitzer et al. 1994) should be tested further; an application of fibre optics to monitor reporter bacteria in porous media was reported by Yolcubal et al. (2000).

Polyphasic approaches based on several, independent assays for comparison with reporter data are highly recommended. Given that many reporters will be designed for specific functional genes and their expression in the future, it must be emphasised how important it is to also monitor total numbers, localisation and survival of the reporter inoculants. An area of future progress for extended incubations is clearly the selection of reporter strains with good survival, typically native strains from the indigenous soil population. Needless to say, improved molecular techniques, e.g. better broad-host-range plasmid vectors, specific transposons for random or site-directed reporter gene insertions, and new *gfp* variants (both native or derived by mutation) with different fluorescence colours and stability characteristics, will become accessible to many more laboratories in the near future. More strains representing functionally important groups of soil bacteria should be compared as reporters; we need to make generalisations from the model reporter strains to natural populations, as soon as possible.

New designs of specificity for the reporter bacteria, e.g. for broad- and narrow-range activation by organic pollutants, must be developed further; one example is that of aromatic pollutant degradation (benzene derivatives) mentioned above, where specificity as determined by the transcription activators such as NadR, XylR, XylS, DmpR is prone to molecular modification. Alternatively, combinations and simultaneous use of several reporters showing different signal specificities may be useful and provide a better overview of toxicity and biodegradation patterns in soil samples. Until reporter specificities are better defined, careful control experiments should always be included in soil systems such as illustrated by the work of Heitzer et al. (1998) who used a *P. fluorescens* HK44 reporter to monitor naphthalene bioavailability and catabolism in aqueous extracts from fuel hydrocarbon-contaminated soil; the authors discovered that non-inducing

organic solvents such as the BTEX compounds toluene and xylene also caused a significant bioluminescence increase. It was found that the solvent exposure led to changed fatty acid synthesis pattern, which in turn affected the bioluminescence reaction because the aldehyde supply for the luciferase was affected.

Some of the latest and most fascinating applications of reporter bacteria have addressed the complicated plant-bacterial, fungal-bacterial or even bacterial cell-cell interactions in soil systems, where most specific molecular signals may be involved in recognition, surface attachment, antagonism, etc. The first, well-known example is that of plant signals (flavonoid compounds) controlling early stages of the legume-*Rhizobium* symbiosis via activation of the bacteral *nod* genes; a *Rhizobium* reporter based on a *nodC-lacZ* fusion was constructed (Bolanos Vasquez and Warner 1997) to study activation by six different flavonoids from host plants (bean). Recently, however, the identification of new, specific environmental signals regulating bacterial growth and activity in the rhizosphere has become feasible with reporter techniques that can sort out activated gene promoters under in vivo conditions. A number of different reports (Rainey 1999; Timms-Wilson et al. 2000; Allaway et al. 2001; Marco et al. 2003) have thus described reporter systems to identify specific rhizosphere-activated promoters in both *Rhizobium* and *Pseudomonas* spp. Briefly, a promoterless reporter is fused into a host strain and the activated host cells are subsequently recovered to identify the promoter control by gene sequencing at the site of reporter insertion. Representing one such example, a *Rhizobium* reporter with promoterless *gfp* captured rhizosphere-activated promoters controlling syntheses of thiamine and cyclic glucans or surface growth-activated promoters controlling methionine synthesis or putrescine uptake, respectively (Allaway et al. 2001).

There has also been recent research using *Pseudomonas* reporter bacteria to study gene-regulating signals from plant-pathogenic "microfungi" (Oomycota). A major interest is here to study the molecular mechanisms of hyphal colonisation and antagonism, supporting the *Pseudomonas* bacteria in plant-protecting biological control. Using *P. fluorescens* strain F113, Smith et al. (1999) found that *Pythium ultimum* released a molecular signal, which down-regulated *rrn* promoters (ribosomal RNA synthesis) in the reporter strain and thus controlled the cellular growth rate. In another study, Lee and Cooksey (2000) found that hyphal colonisation of *Phytophthora parasitica* by a *P. putida* led to activated promoters controlling ABC transporter proteins.

Finally, a role of bacterial cell–cell communication including the new discoveries of cell density-dependent gene regulation in a large number of bacteria has also been indicated in soil and rhizosphere systems (see Chapt. 17 in this volume). A fraction (ca. 40%) of *Pseudomonas* spp.

colonising plant roots was thus reported by Elasri et al. (2001) to produce *N*-acyl-L-homoserine lactone (AHL) molecules, serving as bacterial cell–cell communication signals; interestingly, the AHL production appeared to be more common among plant-associated than among soil-borne pseudomonads. *P. chlororaphis*, *P. fluorescens* and *P. putida* were among the AHL producers, but may actually produce different AHL molecules.

As pointed out in this review, a large number of reporter bacteria are presently available to target both nutrient and pollutant compounds and their degradation in soil. In addition, new signals and promoter controls involved in bacterial host recognition, surface colonisation and other cellular interaction have been described using reporter bacteria. It is safe to say that the new reporter technologies have allowed us, for the first time, to look into the mechanisms controlling growth and specific gene expression within single cells in their natural soil environment.

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17 Reporter Gene Technology in Soil Ecology; Detection of Bioavailability and Microbial Interactions

Mette Burmølle, Lars Hestbjerg Hansen, Søren J. Sørensen

17.1 Introduction

The overall purpose of many microbiological studies in soil is to describe the in situ activity of the indigenous soil bacteria. The aim of such studies is often to evaluate the physical and chemical conditions perceived by a bacterial cell while present in soil. Revealing this may be extremely difficult and sometimes even impossible, mainly because of the heterogeneity of the soil environment. Physical and chemical conditions such as water and oxygen content, and presence of nutrients and organic matter, may vary widely even within very small distances. Also organic compounds produced by the microorganisms in the soil, for instance inhibiting compounds or communication signals, may show a large spatial variation.

Using conventional analytical methods, the presence of anthropogenic compounds in a soil habitat can be measured. However, these methods require extraction of the compound from a certain amount of homogenised soil, and such methods tend to overestimate the availability of some compounds, which usually exist in an insoluble form in the soil (Chaudri et al. 1999). Additionally, the estimated concentration will be an average concentration of the compound in the soil due to the homogenisation. Microsensors are useful in relation to measurements in sediment microhabitats, as they are introduced into local areas of the sediment (Revsbech et al. 1980; Christensen et al. 1994; Lüdemann et al. 2000). They therefore may provide an indication of the conditions to which the bacteria are exposed in this bacterial habitat. However, microsensors are fragile, not suitable for use in heterogeneous environments, and may not always be sufficiently sensitive. Therefore, microsensors are less suited for use in soil. Thus, none of these methods are suitable tools to evaluate to which compounds the soil bacteria are actually exposed and whether those compounds are perceived and responded to by the bacterial cells. An alternative method for revealing this is by use of whole-cell biosensors.

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In this chapter, we present the most commonly used reporter genes and discuss the methods for detection of the reporter gene products, when expressed in soil. Additionally, the use of whole-cell biosensors in detection of bioavailability and microbial interactions will be discussed. We hope to provide the reader with an overview of reporter gene technology applied in soil studies and to illustrate the usefulness of whole-cell biosensors in studies of soil ecology.

17.2 Reporter Genes

A biosensor is a living organism, expressing a measurable product in the presence or absence of the investigated compound or condition. The measurable product is encoded by a reporter gene. Usually, the biosensor is genetically manipulated to contain the reporter gene, for instance in fusion to a regulated promoter; alternatively, the reporter gene is naturally present in the organism. An impact of the detected compound or condition on the biosensor is required for reporter gene expression; thus only conditions affecting the biosensor will be detected and responded to. Therefore, biosensors are very useful tools for detecting bioavailable concentrations of compounds in complex environments such as soil.

A wide range of whole-cell biosensors have been developed and used as tools to qualitatively and quantitatively identify the presence of different compounds or conditions in environmental samples (Hansen and Sørensen 2001). Biosensors have proven useful in detection of toxicity in samples, and therefore biosensor assays are potential candidates to replace conventional analytical methods. Moreover, biosensors have also been used in studies of microbial ecology and gene expression in complex environments (for a review, see Hansen and Sørensen 2001).

Reporter gene systems can be divided into several groups based on the nature of the product expressed. The most commonly used reporter genes in soil studies are *luxCDABE*, *lacZ*, *gfp* and *inaZ*. The properties of these reporter genes are described below and summarised in Table 17.1.

17.2.1 Reporter Genes Encoding Luciferases

Genes encoding light-emitting products are frequently used in biosensor constructs. Bacterial versions include the *luxCDABE* operon from *Vibrio fischeri*, the *luxCDABFE* operon from *Photobacterium leiognathi* and the luciferase coding *luxAB* genes from *Vibrio harveyi*. The *luxAB*-encoded

Table 17.1. Properties of reporter genes used in whole-cell biosensors (– = not applicable, x = little/low, xx = medium, xxx = much/high)

	luxCDABE	*lacZ*	*gfp*	*inaZ*
Sensitivity	xxx	xxx	xx	xxx
Oxygen requirement	yes	no	yes	no
Co-factor independency	x	xxx	xxx	xxx
Expression at low metabolic activity	x	xx	xx	xx
In situ detection	xx	–	xxx	–
Single-cell detection	x	x	xxx	–
Handling ease	xxx	x	xxx	xx
Cell disruption necessary	No	Yes	No	No
Low-cost	xx	xxx	x	xxx

luciferase is a heterodimer composed of two different subunits. It catalyses the oxidation of $FMNH_2$ and a long fatty acid aldehyde in the presence of molecular oxygen. The aldehyde is subsequently regenerated by a multienzyme reductase complex, which is encoded by *luxCDE* (Elasri and Miller 1998). Thus, no substrate addition is required when the *luxCDABE* operon is used and light can be measured in vivo.

The measurement of light is carried out very quickly in a luminometer (Tauriainen et al. 1999) or a scintillation counter (Selifonova et al. 1993). In many studies, fibre optic technology has been used to detect light emission from biosensors containing the *lux* gene (Heitzer et al. 1994; Corbisier 1997; Matrubutham et al. 1997). Typically, cells are immobilised in, for instance, an alginate gel which is placed on, or close to, optic fibres. By connecting the light-guiding optic fibres to a photomultiplier and a recorder, light emission from biosensor cells can easily be quantified.

The main advantage of the *lux* reporter system is that visualisation of the light from the luciferase can be carried out without disruption of the bacterial cells which makes *lux* genes suitable for rhizosphere studies (Kragelund et al. 1997; Jensen and Nybroe 1999). Even if only *luxAB* is used, the aldehyde (the substrate for the light reaction) can be added externally without disrupting the spatial distribution of the bacteria. This can prove particularly advantageous when monitoring distributions of biosensor bacteria in situ. Kragelund and coworkers used a *luxAB* biosensor responding to phosphate starvation, to report phosphate limitation in the rhizosphere of barley (Kragelund et al. 1997; Jensen and Nybroe 1999). A root-colonising bacterium, *Pseudomonas fluorescens*, was allowed to colonise the roots of barley in sterile soil. Thereafter, the roots were exposed to aldehyde vapours and examined using a photonic camera. The roots were brightly luminescent but, after subsequent addition of a phosphate buffer, luminescence

was repressed. This was done without disassociating the bacteria from the roots. The detection limits of advanced photographic detectors allow detection of luminescence originating from aggregates of a few bacteria or even of single cells (Rattray et al. 1995), even though the resolution needs improvement.

Eukaryotic versions of reporter genes which encode light production have also been used in biosensor constructs. These are the luciferase genes, *luc* genes, from the firefly (*Photinus pyralis*) or click beetle (*Pyrophorus plagiophthalamus*; Tauriainen et al. 1998, 1999; Willardson et al. 1998).

Luciferase-based biosensors are either used in situ or alternatively incubated in soil extracts or soil pore water followed by luminescence measurements (Chaudri et al. 1999; Hansen and Sørensen 2000; Porteous et al. 2000; Rasmussen et al. 2000; Vulkan et al. 2000; Flynn et al. 2003). When applying the luciferase-encoding genes as reporters in soil or soil extracts it is necessary to ensure that luminescence is not quenched by soil particles (Rasmussen et al. 2000). Additionally, it should be noted that production of light is an energy-demanding process, and therefore the light output is directly affected by the physiological state of the biosensor cells (Jansson 2003). This is especially important to consider in studies where the biosensor is inoculated in bulk soil for a longer period of time and may become energy deprived. In these cases, it could be advantageous to choose a reporter gene of which expression does not require a metabolic activity as high as that required for the luciferase-encoding reporter genes.

17.2.2 Reporter Genes Based on Chromogenic Detection

The *Escherichia coli lacZ* gene encoding β-galactosidase is also widely used as a reporter gene in biosensors. This reporter gene is extremely reliable which is mainly caused by the simplicity of the *lacZ* gene and its product, β-galactosidase, as well as by the fact that the *lacZ* gene is probably the best-studied reporter gene available. β-Galactosidase can be produced under most circumstances, i.e. it does not require oxygen as do both *luxCDABE* and *gfp* or any other special growth-dependent cofactors (like $FMNH_2$), and it also requires less metabolic activity than *luxCDABE*. The detection of β-galactosidase relies mainly on extraction of the protein from the biosensor cells and a subsequent β-galactosidase assay (Miller 1972). This requires more labour than the direct detection of *lux* and *gfp* products which are both based on photometric detection of emitted and fluorescent light, respectively. However, the β-galactosidase assay provides reliable quantitative responses and, unlike many other reporters, the detection of β-galactosidase does not require expensive equipment.

β-Galactosidase catalyses the reactions of a variety of substrates to different coloured products which are easily detectable. Additionally, some substrates can be converted into products which are oxidised at an electrode, resulting in electrochemical signals that can be measured (Biran et al. 2000).

The *lacZ* gene is not used as frequently in soil studies as the *lux* and *gfp* genes. The reason for this may be that the β-galactosidase measurement requires disruption of the cells and does not allow simple single-cell detection. However, by the use of β-galactosidase-specific fluorescent antibodies, single-cell analysis of *lacZ* expression in soil is possible by flow cytometry (Koch et al. 2001). Biosensors containing a *lacZ* reporter fusion can be inoculated in the soil followed by extraction and disruption of cells, and finally β-galactosidase measurement (Van Overbeek et al. 1997; Højberg et al. 1999; Biran et al. 2000).

Reporter genes like *xylE* (encoding catechol 2,3-dioxygenase), *phoA* (encoding alkaline phosphatase), *uidA* (encoding β-glucuronidase, the GUS reporter system) and *cat* (encoding chloramphenicol acetyl transferase) can be assayed with chromogenic substrates (Miller 1992). They are all potential reporter genes in bacterial biosensor constructs, but they are used less frequently than *lacZ*.

17.2.3 Reporter Genes Encoding Fluorescence

Many recent studies have used the expression of the *gfp* gene, encoding a green fluorescent protein (GFP), as a marker of bacteria introduced into different environments, including soil and rhizosphere. The *gfp* gene was isolated from the jellyfish *Aequorea victoria* and encodes a stable, green fluorescent protein (Chalfie et al. 1994). Detection of GFP requires only excitation by light (typically 395 or 488 nm depending on the GFP variant; Cormack et al. 1996; Errampalli et al. 1999) followed by measurement of emitted light. It is therefore not limited by the availability of substrates, like the detection of both β-galactosidase and the eukaryotic luciferases. Bacteria containing GFP can be detected at the single-cell level. The detection of single cells relies on techniques like epifluorescence microscopy (Joyner and Lindow 2000; Casavant et al. 2002, 2003) or flow cytometry (Hansen et al. 2001; Burmølle et al. 2003). Spatial information can be obtained at high resolution even from GFP-containing bacteria in environmental samples by using confocal laser scanning microscopy (CLSM; Bringhurst et al. 2001; Steidle et al. 2001). An example of visualisation of *gfp*-expressing biosensor cells at the single-cell level by CLSM is presented in Fig. 17.1.

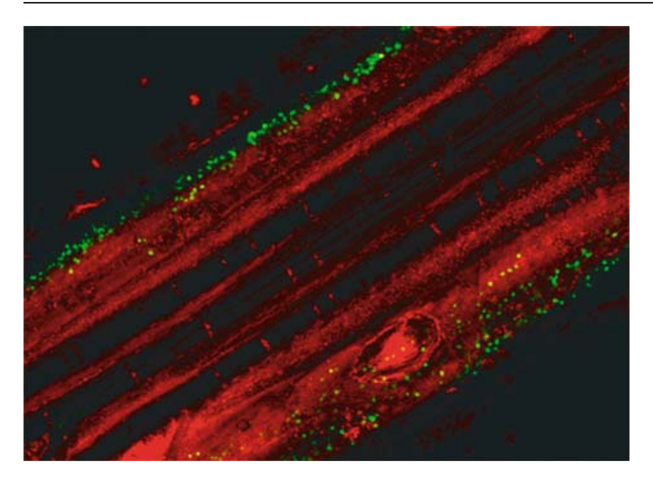


Fig. 17.1. Visualisation by confocal laser scanning microscopy of *gfp*-expressing biosensor cells on a wheat straw. A biosensor detecting bacterial communication signals, *N*-acylhomoserine lactones, was introduced onto the straw (Burmølle et al., unpubl. res.)

In recent years, *gfp* has been used more and more frequently as a reporter gene, especially in environmental studies. This is mainly due to the ability of GFP detection at the single-cell level, although this detection requires expensive and advanced equipment. However, single-cell detection provides a powerful tool for in situ studies of complex habitats such as rhizosphere (Bringhurst et al. 2001; Steidle et al. 2001) and bulk soil (Hansen et al. 2001; Burmølle et al. 2003). In such studies, *gfp*-based biosensors are usually incubated in bulk or rhizosphere soil and thereafter analysed for green fluorescence by one or several of the above-mentioned methods. Alternatively, *gfp*-expressing biosensors can be incubated in soil extracts and then be analysed by fluorescence measurements. However, in such applications, the sensitivity of the *gfp* gene is low compared to the *lux* and *lacZ* reporter systems.

The presence of oxygen is required for the GFP protein to fold properly and fluoresce (Tsien 1998). Thus, in oxygen-limited conditions, which occasionally are present in soil, GFP-expressing biosensors may not be suitable. In our laboratory, however, we have not experienced this feature of GFP to be a problem during applications of *gfp*-based biosensors in soil. Additionally, if the biosensor cells are handled in samples prior to analysis, for instance during extraction from the soil, the sample will become aerated and the GFP present in the cells will fold up and fluoresce.

Modified *gfp* Variants

GFP has been genetically modified for several purposes. Some versions of GFP have reduced half-lives compared to the natural GFP, they are known as unstable GFP variants (Andersen et al. 1998). This property can be used in biosensor studies of the soil environment. When residing in soil for several days, some leakiness of the promoter, inducing *gfp* expression, results in a low, constant GFP production. The natural version of GFP is very stable (Tombolini et al. 1997) and will over time accumulate in the cells, which then eventually become green fluorescing. In such cases, it may be difficult to distinguish this background fluorescence from that caused by the detected compound. One way to circumvent this problem is to use an unstable *gfp* version, of which the half-life is markedly reduced (half-life of approximately 40 min; Andersen et al. 1998). Such unstable *gfp* variants are also suitable for studies of transient gene expression.

The excitation and emission wavelength peaks of GFP have also been modified. The FACS-optimised GFP version, GFPmut3, where the excitation wavelength was shifted to 488 nm (Cormack et al. 1996), is well suited for analysis by flow cytometry, epifluorescence microscopy and CLSM. Other variants have shifted emission and excitation peaks, for instance the yellow fluorescing and the blue fluorescing proteins. These have successfully been applied for visualisation of various bacterial species in the rhizosphere (Stuurman et al. 2000). Some native proteins have similar properties, for instance *DsRed* from the coral *Discosoma* sp. that encodes a red fluorescing protein (Gross et al. 2000). The availability of reporter genes fluorescing in different colours offers the opportunity to study the expression of different genes simultaneously. Perhaps more importantly, these genes are useful as constitutively expressed strain markers, in combination with *gfp* as reporter gene, when the emission peaks of those proteins can be distinguished. This is for instance the case for *gfp* and *DsRed*. Steidle et al. (2001) used this approach when studying bacterial communication in the rhizosphere. Constitutively expressed *DsRed* was used in visualisation and localisation of biosensor cells and *gfp* induction detected bacterial communication. In this study, green and red fluorescence was detected by CLSM. Fluorescence from *gfp* and *DsRed* can also easily be detected and well distinguished by flow cytometry (Maksimow et al. 2002). Flow cytometric analysis of such biosensors enables detection of the specific compound and quantification of the fraction of induced biosensors simultaneously.

Another reporter gene resulting in red fluorescence is the *cobA* gene from *Propionibacterium freudenreichii*. This gene encodes a uroporphyrinogen III methyltransferase. When over-expressed, *cobA* causes accumulation of a trimethylated compound that emits strong red fluorescence, which is detected by exposure to ultraviolet or blue light (Wildt and Deuschle 1999).

17.2.4 Reporter Genes Encoding Ice-Nucleation Activity

The *inaZ* reporter gene is in general less used than those mentioned above. However, in soil studies, *inaZ* has been applied several times. The *inaZ* gene originates from *Pseudomonas syringae* (Wood et al. 1997) and encodes the ice-nucleation protein, InaZ, that promotes ice-nucleation activity. This activity is measured in detection of *inaZ* expression (Orser et al. 1995). In practice, droplets of the biosensor suspension are spotted onto foil floating in an alcohol bath at a temperature below 0 °C. Because expression of *inaZ* promotes droplet freezing, the fraction of frozen droplets detected is a measure of the level of *inaZ* expression (Lindgren et al. 1989). The usual application of *inaZ*-based biosensors in soil studies is in situ incubation, followed by extraction and measurements of ice-nucleation activity; *inaZ* has been used as reporter gene in environmental studies of *N*-acylhomoserine lactone (AHL)-mediated quorum sensing (Wood et al. 1997; Pierson et al. 1998) and availability of sucrose and tryptophan (Jaeger et al. 1999) and iron (Marschner and Crowley 1996; Loper and Henkels 1997) in the rhizosphere.

17.3 Whole-Cell Biosensors

In general, whole-cell biosensors can be divided into three groups dependent on the mechanism of reporter gene regulation; non-specific, semi-specific (or stress-induced) and specific.

Non-specific biosensors usually rely on constitutively expressed reporter genes to evaluate or sense the presence of toxic compounds. In several studies, the constitutively expressed *luxCDABE* operon from *V. fischeri* was used to detect toxicity in soil (Table 17.2). In such toxicity assays the *lux* genes are usually highly expressed in cells that are not exposed to toxic effects in the media. A measurement of the decrease in light production from these cells will therefore reflect an inhibitory effect of the sample added to the bacteria. Such measurements are non-specific because any condition that inhibits bacterial growth will reduce the production of light from the biosensor strain. Conditions like pH, salinity, metals and presence of xenobiotics and many other compounds are potential inhibitors of light production. This makes it very hard to assess the nature and the gravity of the toxicity problem. Nevertheless, these non-specific sensors have been used to determine the toxicity level in soil of metals and xenobiotics (Table 17.2). Additionally, non-specific *lux*-based biosensors have proven to be useful in evaluating the metabolic activity of bacteria present in soil and rhizosphere

Table 17.2. Non-specific biosensors used in soil or rhizosphere studies

Compound or condition sensed	Reporter genes	Host strain[a]	Environment	Reference
2,4-Dichlorophenol	*luxCDABE*	*E. coli*	Soil extract	Beaton et al. 1999
Alkylbenzene sulfonate	*luxAB*	*Nitrosomonas europaea*	Soil slurry and extract	Brandt et al. 2002
BTEX compounds	*luxCDABE*	*P. fluorescens*	Sediment extract	Sousa et al. 1998
C	*luxCDABE*	*P. fluorescens*	Soil extract	Standing et al. 2003
CPC, TPC, PCP	*luxCDABE*	*E. coli, P. fluorescens*	Soil extract	Tiensing et al. 2002
Cu	*luxCDABE*	*E. coli, P. fluorescens*	Soil pore water	Vulkan et al. 2000
Cu, Cd and PCP	*luxCDABE*	*Rhizobium leguminosarum, P. fluorescens*	Soil extract	Palmer et al. 1998
Metabolic activity	*luxCDABE*	*Enterobacter cloacae*	Rhizosphere	Rattray et al. 1995; de Weger et al. 1997
Metabolic activity	*luxAB*	*P. fluorescens*	Soil, in situ	Unge et al. 1999
Metabolic activity	*luc*	*Arthrobacter chlorophenolicus*	Soil, in situ	Elvang et al. 2001
PAH toxicity	*luxCDABE*	*E. coli*	Soil extract	Gu and Chang 2001
Pollutant stress	*luxCDABE*	*P. fluorescens*	Root extract	Porteous et al. 2000
Zn	*luxCDABE*	*E. coli, P. fluorescens*	Soil pore water	Chaudri et al. 1999

[a] *E. = Escherichia, P. = Pseudomonas*

(Table 17.2). Examples of applications of non-specific whole-cell biosensors are described in Chap. 16.

The semi-specific biosensors contain a reporter gene fused to a stress-regulated promoter and are therefore induced by various conditions stressful to the biosensor organism. These include antimicrobial agents, genotoxins, oxidative stress and ultraviolet radiation (Hansen and Sørensen 2001; see Chap. 16). This type of biosensors has so far not been used in studies of the soil environment. However, in our laboratory, we have recently applied such biosensors to estimate genotoxicity of contaminated soils (Norman et al. 2005).

In contrast to both the non-specific and the semi-specific biosensors, very specific responses to different compounds are produced by a variety of biosensors. Similar to the stress-induced biosensors, the specific biosensors rely on genetic engineering to generate transcriptional fusions between inducible promoters and reporter genes. These constructs usually also

contain regulatory genes encoding repressor or activator proteins that are regulated by the detected compound. In some cases, as for instance the Hg-sensitive biosensor, the repressor protein changes to an activator when interacting with the compound, see Fig. 17.2. The range of compounds detected by specific biosensors is limited only by the discovery of genes regulated according to environmental conditions.

Specific biosensors are excellent tools for investigations of expression of selected genes in complex environments such as soil. As this gene expression reflects an impact on the biosensor by a compound, information is provided on which compounds the biosensor was exposed to in the soil. Therefore, biosensors are useful in estimations on whether or not a compound present in the soil is perceived by the biosensor, in such a way that it results in a change in bacterial gene expression. Specific biosensors have been developed to detect a range of different compounds and conditions, including xenobiotics, metals, nutrient status and compounds mediating communication (Table 17.3).

If the investigated compound is only present at low concentrations and/or in short periods of time in the environment, it may not be possible to detect

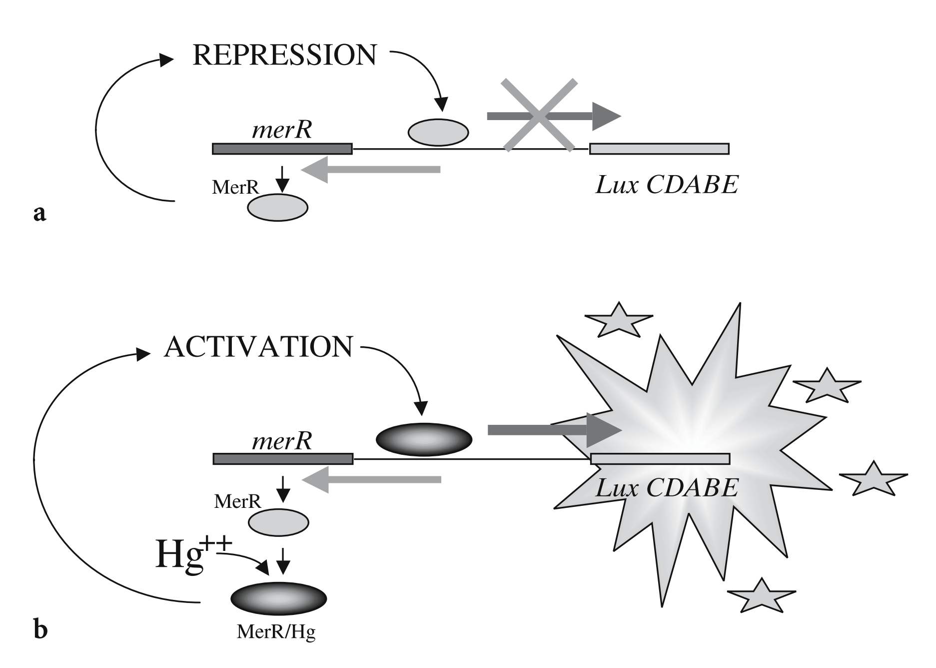


Fig. 17.2. Induction of a Hg-*luxCDABE* biosensor. **a** In the absence of mercury, the *merR* gene product, MerR, acts as a repressor protein, preventing transcription of the *lux* genes. **b** In the presence of Hg^{2+}, the MerR protein interacts with Hg^{2+} and serves as an activator of *lux* gene transcription. Subsequently, measurable quantities of light are produced

Table 17.3. Specific biosensors used in soil or rhizosphere studies

Compound or condition sensed	Gene fusion	Host strain[a]	Environment	Reference
4-Chlorophenol	*bphA::lacZ*	*P. fluorescens*	Rhizosphere[b]	Brazil et al. 1995
Anaerobicity	P_{ANR}-*lacZ*	*P. fluorescens*	Soil and rhizosphere[b]	Højberg et al. 1999
Antimony	P_{ars}-*luxAB*	*E. coli*	Soil extract	Flynn et al. 2003
Arabinose	P_{BAD}-*gfp*	*Enterobacter cloacae*	Soil and rhizosphere[b]	Casavant et al. 2002
Arsenite and Hg	P_{ars}-, P_{mer} -*lucGR*	*P. fluorescens*	Soil extract and slurry	Petanen and Romantschuk 2003
C	P_{fic}-*lacZ*	*P. fluorescens*	Soil[b]	Koch et al. 2001
C	Tn5-*lacZ*[c]	*P. fluorescens*	Soil[b]	van Overbeek et al. 1997
Cd	P_{antZ}-*lacZ*	*E. coli*	Soil[b]	Biran et al. 2000
Cu	*cop::luxAB*	*P. fluorescens*	Soil slurry	Perkiomaki et al. 2003
Cu	*cop::luxAB*	*P. fluorescens*	Soil extract	Tom-Petersen et al. 2001
Fe	P_{pvd}-*gfp*	*P. syringae*	Rhizosphere[b]	Joyner and Lindow 2000
Fe	P_{pvd}-*inaZ*	*P. fluorescens*	Soil, rhizosphere[b]	Marschner and Crowley 1996; Loper and Henkels 1997
Galactosides	P_{melA}-*gfp*	*Sinorhizobium meliloti*	Rhizosphere[b]	Bringhurst et al. 2001
Hg	$P_{merTPAD}$ -*luxCDABE*,	*P. putida*	Soil extract	Hansen and Sørensen 2000
Hg	$P_{merTPAD}$ -*luxCDABE*	*E. coli*	Soil extract	Rasmussen et al. 2000
Lysine	*davT::luxCDABE*	*P. putida*	Rhizosphere[b]	Espinosa-Urgel and Ramos 2001
N	Tn5-*luxAB*[c]	*P. fluorescens*	Soil and rhizosphere[b]	Jensen and Nybroe 1999
N and P	Tn5-*luxAB*[c], P_{P2}-*luxAB*	*P. fluorescens*	Soil extract	Standing et al. 2003
N-acylhomo-serine lactones	P_{luxI}-*gfp*	*E. coli*	Soil[b]	Burmølle et al. 2003
N-acylhomo-serine lactones	*PhzB::inaZ*	*P. aureofaciens*	Rhizosphere[b]	Wood et al. 1997; Pierson et al. 1998

Table 17.3. (continued)

Compound or condition sensed	Gene fusion	Host strain[a]	Environment	Reference
N-acylhomo-serine lactones	P_{cepI}-, P_{luxI}-*gfp*	*P. putida, Serratia liquefaciens*	Rhizosphere[b]	Steidle et al. 2001
Napthalene and salicylate	*nahG::luxCDABE*	*P. fluorescens*	Soil slurry	King et al. 1990
Naphthalene and salicylate	*nahG::luxCDABE*	*P. fluorescens*	Soil extract	Heitzer et al. 1994
Ni	*cnrYXH ::luxCDABE*	*Ralstonia eutropha*	Soil extract	Tibazarwa et al. 2001
Oxytetracycline	P_{tet}-*gfp*	*E. coli*	Soil[b]	Hansen et al. 2001
P	Tn5-*lacZ*[c]	*P. putida*	Soil and rhizosphere[b]	de Weger et al. 1994
P	P_{P2}-*luxAB*	*P. fluorescens*	Rhizosphere[b]	Kragelund et al. 1997
PCB (polychlorinated biphenols)	*Pm::gfp*	*P. fluorescens*	Rhizosphere[b]	Boldt et al. 2004
Sucrose and tryptophan	P_{scrY}-*inaZ*, *aatl::inaZ*	*Erwinia herbicola*	Rhizosphere[b]	Jaeger et al. 1999
Toluene	P_{tbuA1}-*gfp*	*P. fluorescens, Enterobacter cloacae*	Soil and rhizosphere[b]	Casavant et al. 2003
Toluene	P_u-*luc*	*E. coli*	Soil extract	Willardson et al. 1998

[a] *P. = Pseudomonas, E. = Escherichia*
[b] Study performed in situ
[c] Promoter not identified

this compound by a “traditional” specific biosensor based on a fusion between a regulated promoter and a reporter gene. This is mainly due to the fact that the induction level of reporter gene expression may not exceed the background fluorescence caused by promoter leakiness. Additionally, a response produced from a low and/or transient induction may become degraded or diluted during cell division, and thus this response is not detected.

To circumvent this problem, an alternative reporter gene system has been developed. In this system, the investigated compound induces an irreversible recombination event, rendering the reporter gene constitutively induced (Casavant et al. 2002, 2003). Hence, the reporter gene regulation relies on an on/off mechanism unlike traditional biosensors, in which the

intensity of reporter gene expression reflects concentrations of the inducing compound. By use of the "recombination-biosensors", presence of arabinose (Casavant et al. 2002) and toluene (Casavant et al. 2003) has been detected in the rhizosphere. These biosensors are very sensitive to the specific compounds, although some promoter leakiness was observed. The recombination event requires more enzyme activity than induction of traditional biosensors, therefore the biosensors must be metabolically active to promote reporter gene expression. The consequence of this is that these biosensors are only useful in environments of high nutrient availability, such as rhizosphere. In fact, Casavant et al. (2003) showed that the toluene-sensitive biosensor lost the ability to respond to the presence of toluene after residing in bulk soil for more than 24 h. Nevertheless, these new biosensors are interesting supplements to traditional specific biosensors.

A detailed description of applications of specific biosensors in the soil environment is given in Chap. 16. In the present chapter, we will focus on the use of specific whole-cell biosensors in estimations of bioavailability (Sect. 17.4) and detection of microbial interactions (Sect. 17.5).

17.4 Bioavailability

The biosensors listed in Table 17.3 are potential detectors of the bioavailability of different chemical compounds or specific environmental conditions. This is extremely useful in a complex environment such as soil. As mentioned in Sect. 17.1, it is very hard to measure which compounds bacteria are exposed to in soil. The main reason for this is that bioavailability in soil is very difficult to estimate, and often assumptions must be made even though they are known to be imprecise. In soil, the bioavailable fraction of metals has been defined as the fraction which is soluble in water (Rasmussen et al. 2000) or in 0.1 N $Ca(NO_3)_2$ (Weissenhorn et al. 1995) and it is generally determined after dilution or extraction (Paton et al. 1997; Willardson et al. 1998; Beaton et al. 1999; Rasmussen et al. 2000). This may alter the solubility, and thereby the bioavailable fraction of the compound. Or, the compound may be diluted to concentrations below the detectable limit. One way to avoid this dilution/extraction step was shown in a study by Chaudri et al. (1999) who used a Rhizon soil moisture sampler, consisting of porous inert plastic tubes through which the soil pore water was extracted. Subsequently, the acute Zn toxicity in soil pore water was evaluated by use of a *Pseudomonas putida* harbouring a constitutively expressed *luxCDABE* cassette. This soil moisture sampler was also used by Vulkan et al. (2000) to determine Cu speciation and availability.

It should be emphasised that, despite the estimations described above, bioavailability remains an undefined parameter which depends on the organism used and the experimental methods applied. Indeed, different strains had remarkably different sensitivities to metals (Paton et al. 1995, 1997). The choice of reporter strain should therefore reflect the aim of the investigation and the environment in which the experiment is performed. Since *E. coli* is not native to soil, its relevance as an environmental biosensor is questionable and the use of more environmentally relevant biosensor bacteria is preferable.

Concerning estimations on toxicity levels of bioavailable contaminants it is important to bear in mind that those compounds may cause additive, synergistic or antagonistic interactions (Preston et al. 2000). This means that the impact of a certain toxic compound to a soil organism is not solely determined by the bioavailable fraction of this compound, but also by the presence of other compounds.

17.4.1
Use of Biosensors To Measure Bioavailability of Metals in Soil

Whole-cell biosensors are useful in estimations of bioavailability in soil. One application is to evaluate how changes in physical and chemical parameters affect bioavailability of metals. Recently, Tom-Petersen et al. (2004) used a bioluminescent, Cu-specific reporter constructed in *P. fluorescens* (Tom-Petersen et al. 2001) to determine the biological availability of Cu in soil water extracts as affected by time and moisture conditions. These authors found that less than 1% of the soluble Cu was bioavailable. The total Cu concentration as well as the bioavailable Cu concentration decreased with time but bioavailability (bioavailable [Cu]/total soluble [Cu]) tended to increase with time. Furthermore, drying of the soil decreased bioavailability (Tom-Petersen et al. 2004). The same Cu-specific reporter strain has been useful in studies addressing the ability of organic compounds to affect bioavailability of this metal. Tom-Petersen et al. (2001) demonstrated that amendment of agricultural soil with straw and manure increased the soluble Cu pool in the soil but lowered its bioavailability, possibly due to chelation of the Cu by the organic matter.

Biosensors react on substrates entering bacterial cells and subsequently produce a measurable response, i.e. a protein. They report expression from promoters which are usually responsible for expressing genes encoding products such as metal resistance proteins or enzymes involved in degradation pathways. Contaminated soil, inducing a response to an Hg biosensor, will therefore also contain enough Hg to favour expression from *mer* resistance genes (*merTPCA*) within bacteria living there. Expression of these

genes results in a reduction of Hg^{2+} to elemental Hg, which evaporates from the soil, resulting in a decrease in bioavailable Hg. Biosensors are therefore ideal for investigating bioavailability of compounds and the physiology and gene expression of microorganisms in complex environments.

Bioavailable Hg was measured (by a *merR*-P_{mer}-*luxCDABE* biosensor, see Fig. 17.2) in extracts from soil microcosms contaminated with different amounts of the toxic heavy metal (Rasmussen et al. 2000). The study showed a cascade of effects resulting from the contamination. First a rise in the number of Hg-resistant bacteria was observed in the most contaminated soil microcosms, accompanied by a decrease in bacterial diversity. This resulted in a decrease in bioavailable Hg, probably caused by reduction of Hg^{2+} to elemental Hg that evaporated from the microcosms. As the bioavailable Hg disappeared, the number of Hg-resistant bacteria and the bacterial diversity returned to normal levels. This study illustrates that biosensors can report the expression of genes in the environment induced by a bioavailable fraction of a compound.

17.4.2 In Situ Versus Extract Measurements of Bioavailability

A way to circumvent the problems arising from dilution and extraction of compounds in soil is by examining gene expression in situ. In studies performed in situ (Table 17.3), the whole-cell biosensors are introduced into the environment for a period of time ranging from hours to weeks. Thereafter, biosensors are recovered by extraction or, for rhizosphere studies, separation of the root and associated bacteria from the soil. Reporter gene expression of biosensors is then analysed.

It is worth noting that in situ investigations can be performed by use of biosensors harbouring the reporter genes described in this chapter: *lux*, *lacZ*, *gfp*, and *inaZ*. When choosing the biosensor organism, it should be remembered that in situ studies require incubation of the biosensor strain in soil, usually for several days. Therefore, this strain must be able to survive in the soil environment. The survival of the biosensor strain may be significantly increased when it is isolated from the investigated environment. From Tables 17.2 and 17.3 it is clear that many host strains of biosensor constructs are indigenous soil or rhizosphere bacteria.

Apart from the elimination of compound extraction and dilution, in situ studies confer several other advantages. First of all, precise information can be obtained on the spatial distribution of compounds. For example, bioavailable tryptophan and sucrose have been shown around roots of grass (Jaeger et al. 1999). Another advantage of studying the soil environment in situ is that high local concentrations of a specific compound present in a low

average concentration are detectable. Due to the heterogeneity of soil, many compounds may be present at high concentrations in a few microhabitats and absent from others. This may be the case for compounds produced by the soil bacteria, for instance growth- or activity-related compounds such as antibiotics and communication signals, as described in the next section. When extracted, the concentrations of such compounds may be far below the detection limit, but, by in situ detection followed by single-cell analysis, the presence of such compounds in bulk soil can be detected.

It is generally believed that the in situ biosensor measurement is a non-disturbing experimental method, where the introduction and presence of biosensor cells have no impact on the system to be studied. However, this is not the case. Conditions such as water content, spatial bacterial distribution and compactness of a soil microcosm are inevitably affected when introducing the biosensor cells into a soil or rhizosphere environment. Additionally, the presence of these biosensors may also change the balance in the microcosm, dependent on the ability of colonisation and survival of the biosensor organism. The introduced organisms may serve as competitors for space and nutrients or as a nutrient source for the indigenous population.

17.5 Detection of Microbial Interactions

A unique potential of whole-cell biosensors is detection of microbial interactions in the soil environment. These interactions are based on microbial production and excretion of compounds such as antibiotics and communication signals which affect nearby microorganisms. As described above, compounds produced by the soil bacteria are often unevenly distributed and the average concentrations are non-detectable. Even if these compounds were detected, the concentrations would be very low, and it would be questionable whether that was sufficient to impact on the soil bacteria. Therefore, whole-cell biosensors are valuable tools in studies of production of such compounds in bulk soil and rhizosphere.

17.5.1 Production of Oxytetracycline

It has been a scientific dispute for years whether or not antibiotic compounds are produced in the soil environment (Gottlieb 1976; Williams 1982). By the use of biosensor technology, it was possible to develop a tool for detection of production of tetracycline in bulk soil (Hansen et al. 2001).

Expression of the *tetA* gene, conferring resistance to tetracycline, is regulated by the presence and absence of tetracycline via a promoter, P_{tet}, and a repressor protein, TetR. This was exploited in the development of a tetracycline-sensitive whole-cell biosensor. P_{tet} was fused to *gfp* in a plasmid that also contained TetR. An *E. coli* strain harbouring the plasmid was incubated into sterile soil together with spores of *Streptomyces rimosus* that is a known industrial producer of oxytetracycline. Two days after introduction, 28% of the biosensors had accumulated enough GFP to be detected, both by flow cytometry and by epifluorescence microscopy. After 5 days of inoculation, 2.4% of the biosensor population was induced (Hansen et al. 2001).

This is the first study demonstrating detection of antibiotic production in soil in situ and it illustrates perfectly the usefulness of biosensor technology in studies of microbial interactions. Recently, tetracycline production in non-sterile bulk soil, without producer addition, was detected by use of this biosensor (unpublished results). These results strongly indicate that soil microhabitats exist in which a selective pressure for tetracycline resistance is established, favouring the preservation of tetracycline-resistance genes. This is a very important issue to bear in mind in relation to the release of genetically manipulated bacteria in the soil environment, as the resistance genes, which usually serve as markers, are believed to be lost due to the absence of a selective pressure in soil. Also, dissemination of wastes from livestock in the soil environment is likely to contain bacteria with multiple antibiotic resistances. Introduction of resistance genes into the soil environment may contribute to the expansion of the pool of resistance determinants that can eventually spread to pathogenic organisms.

17.5.2 Production of Communication Signals

Bacterial communication is also known as quorum sensing (Fuqua et al. 2001). By sending and receiving communication signals, bacteria regulate gene expression according to the population density present. In many Gram-negative species, quorum sensing is based on production and detection of the diffusible compounds, *N*-acylhomoserine lactones (AHLs; Whitehead et al. 2001). By AHL-mediated quorum sensing, individual cells in a population are able to coordinate their activities and express certain functions only when high bacterial densities are present. These functions include production of light, pigments, enzymes, antibiotics, biotoxins and compounds promoting virulence, and they are all expressed from inducible promoters (Miller and Bassler 2001; Whitehead et al. 2001). Therefore, it has

been possible to develop AHL-detecting whole-cell biosensors to be used in quorum-sensing studies (Wood et al. 1997; Andersen et al. 2001; Steidle et al. 2001; Burmølle et al. 2003). In these biosensors, an AHL-inducible promoter is fused to a reporter gene. They also contain a gene encoding an activator protein that, when bound by AHL, is able to activate reporter gene expression. This leads to a response when AHL compounds are present in the environment. By use of AHL-sensitive biosensors, bacterial communication has been demonstrated to occur in the plant rhizosphere. Wood et al. (1997) showed that isogene *Pseudomonas aureofaciens* strains communicate by AHLs when incubated in the wheat rhizosphere. This study was performed by use of the *phzB-inaZ*-based biosensor, which was also used by Pierson et al. (1998) to show that also non-isogenic strains of *Pseudomonas* spp. were able to produce and detect AHLs in the wheat rhizosphere. Additionally, Steidle et al. (2001) applied *gfp*-based biosensors and single-cell detection, by CLSM, to show AHL-mediated quorum sensing in the tomato rhizosphere between two *P. putida* strains and also between *Serratia liquefaciens* and *Rahnella aquatilis*. Furthermore, by using a biosensor marked chromosomally with the AHL-inducible *gfp* construct, they showed AHL production by the indigenous bacterial community colonising the tomato rhizosphere.

Production of AHL compounds has also been detected in sterile bulk soil (Burmølle et al. 2003), where both the AHL-producing *S. liquefaciens* and an AHL-detecting, *gfp*-expressing biosensor were incubated. After 1 and 3 days, large fractions of the extracted biosensor population consisted of green fluorescing cells (42% and 48%, respectively), detected by flow cytometry. Additionally, we have recently detected AHL production by the indigenous soil bacteria during litter decomposition processes in non-sterile bulk soil (Burmølle et al. 2005).

Studies referred to above strongly indicate that soil bacteria perceive signals providing information on their own and also neighbouring populations. Furthermore, it has been demonstrated that plants secrete substances that mimic bacterial AHL communication activities (Teplitski et al. 2000; Gao et al. 2003). Therefore, soil bacteria may also be able to sense the presence of plants.

17.6 Concluding Remarks

Biosensor technology has expanded much in the past decade. Even though soil is a very complex environment that introduces additional requirements to reporter gene application and detection, many biosensor studies have been performed in soil. Such studies have proven to be excellent tools

revealing compounds and conditions to which soil bacteria are exposed, concerning not only presence of toxic compounds, but also nutrient and oxygen status. By evaluating such factors we better understand the bacterial life in the soil environment, which is very difficult or even impossible to obtain by other methods.

Biosensors are ideal for investigating the physiology and gene expression of microorganisms in complex environments like soil, including interactions between different members of the soil community, like tetracycline produced from *S. rimosus*, nutrients excreted from root tips of plants or bacterial communication.

We have presented several examples of soil studies based on the use of whole-cell biosensors and we believe many more are to come, because reporter genes and detection technologies are in constant development, with some aiming to improve the applicability of whole-cell biosensors in soil. Detection of the presence of multiple compounds in an environment, as sucrose and tryptophan or N, P and C status, can be simplified by use of genes encoding blue, red and yellow fluorescing proteins. As the use of techniques like epi-fluorescence microscopy, flow cytometry and confocal scanning laser microscopy is increasing, so is the resolution at which we can analyse microbial ecology in complex environments.

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18 Marker Genes As Tools To Study Deliberately Released Soil Bacteria

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18.1 Introduction: The Importance of Tagging Microbial Inoculants for Environmental Applications

Soils harbour enormous reservoirs of different microorganisms of which many have not yet been cultivated and characterised. Cultivation-independent techniques open new possibilities to discover and exploit new biotechnological potentials encoded in the soil metagenome (Lorenz et al. 2002; Schloss and Handelsman 2003; Voget et al. 2003; Eggert et al. 2004). Nevertheless, traditional techniques based on the enrichment and isolation of pure cultures have already allowed microbiologists to obtain many microorganisms with potentially beneficial traits, e.g. those that produce antibiotics or insecticides, others that degrade pollutants or mobilise soil bound metals, and those which promote the growth of agriculturally important crops. The exploitation of these potentials and their improvement is one of the key activities in modern biotechnology and it is also becoming increasingly important for environmental engineering.

For industrial production systems, biotechnology research has already resulted in a tremendous output of products and a high commercial success, but the exploitation of soil microorganisms for environmental applications, e.g. to clean up polluted soils or to replace agricultural fertilisers, is still quite limited (Watanabe and Baker 2000; Dua et al. 2002; Morrissey et al. 2002; Sessitsch et al. 2002; Vessey 2003). It is obvious that this discrepancy relates to the fact that industrial fermentation plants work with defined media and highly controlled external parameters supporting the growth and specific gene expressions of the selected organisms, while microorganisms deliberately released into soil face environmental conditions which are highly heterogeneous, variable and mostly uncontrolled.

In order to develop strategies for improving microbial inoculants for environmental applications it is important to understand how introduced

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microorganisms behave in nature and how nature affects their survival and activity. The general properties and genetic potentials, as well as the general regulation of metabolic activities of inoculants, can best be studied with pure cultures in defined growth media under laboratory conditions. However, as suggested by the yet limited success of using microbial inoculants in the environment, and as demonstrated in numerous studies, the actual behaviour of inoculated microorganisms cannot be predicted within this experimental framework. A further step for evaluating and understanding microbial inoculants is to study their fate and activity in a simplified natural substrate, e.g. sterilised soil or in native soil under defined conditions, i.e. in microcosms in the laboratory or greenhouse (Trevors et al. 1990; Angle et al. 1995; England et al. 1995; Schwieger et al. 1997; Hill and Top 1998; Molina et al. 2000; Cavalca et al. 2002).

The most sensitive technique for quantifying the survival of inoculants is, in most cases, their detection by cultivation on appropriate media, even though care has to be taken as some microorganisms lose their culturability after soil inoculation (Alexander et al. 1999; Grey and Steck 2001). Re-isolation by cultivation can easily be applied to studies with pre-sterilised soils, as no members of the indigenous microbial community will interfere. In fact, studies with pre-sterilised soil-matrices can be valuable, e.g., to compare the survival rates of genetically modified microorganisms with their non-modified counterparts (Vahjen et al. 1997). However, they are not applicable for predicting survival rates in the environment, as the indigenous microbial community represents a highly selective component that limits the success of the colonisation of an inoculant, mainly because of substrate competition and protozoan grazing (Schwartz et al. 2000; Clancey et al. 2002; Bennett et al. 2003).

18.2 Genetic Tools for Tagging Inoculants

The study of the fate of inoculants in native soil by cultivation requires that they grow under selective conditions which reduce the growth of other soil microorganisms as these background organisms would decrease the threshold of detection. Unfortunately, in most instances, a cultivation technique that exclusively enriches the inoculant is not available, and other microorganisms, which might be very similar to the inoculant, will also be enriched. To increase the sensitivity, reliability and convenience of a specific detection, it would be desirable if the inoculant could easily be distinguished from those other co-cultivated organisms, preferably with an inexpensive detection technique. Such a combined system would not only be applicable to study the fate of inoculants in microcosms or greenhouses, but also in

field studies in which, to cope with the high variability in the environment, the number of samples will naturally be much higher. Several laboratory studies with soil microcosms have used microbial inoculants with natural antibiotic resistances as phenotypic markers for quantifying their survival, but the lack of a highly specific marker made these detections from soil very difficult (Liang et al. 1982; Weller and Cook 1983; Fredrickson and Elliott 1985; Compeau et al. 1988; Lochner et al. 1991).

The progress needed for a specific marker technology came from molecular bacterial genetics, which provided the tools to tag a selected strain in its genome and, by means of gene expression, also to confer a specific phenotype. Using transposon mutagenesis, bacterial inoculants were tagged with selectable markers (Van Elsas et al. 1986). Transposons are mobile genetic elements which can move between different genetic entities, e.g. from plasmids of one organism to chromosomes of another. These transposons were transferred in the laboratory from *Escherichia coli* donor strains, in which they were constructed and maintained, to various recipient strains (Hernalsteens et al. 1978; Simon et al. 1983). The first construct that was generated for deliberate field releases using such a technique was the soil bacterium *Pseudomonas aureofaciens* with a *lacZY* gene of *E. coli* inserted into the chromosome with transposon Tn*7*, from which the antibiotic resistance encoding genes *dhfr* and *aadA*, originally present, had been removed (Drahos et al. 1986; Craig 1989; Kluepfel et al. 1991). The *lacZY* genes confer the ability to utilise lactose as a sole source of carbon (C). As this property is very rare or unique within soil microbial communities, inoculants with *lacZY* can easily be quantified from soil by cultivation on agar with lactose as a C source. In addition, the identity of the cultivated bacterial colonies can be confirmed with a colour assay using the substrate conjugate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) which releases a blue compound after cleavage by the *lacZ*-encoded β-galactosidase.

As recombinant DNA-technology was used to remove the antibiotic resistance genes and to insert the *lacZY* gene cassette into Tn*7*, constructs such as *P. aureofaciens* Ps3732RN Lac$^+$, and later other *Pseudomonas* strains tagged with the same system, were classified as "genetically engineered" and, thus, field releases required permission by the respective public authorities. In fact, these constructs were among the first genetically engineered bacteria to be deliberately released, and the potential environmental and human health risks of these ventures were thoroughly analysed (Drahos et al. 1988; Ryder 1994).

In other studies, the non-modified transposon Tn*5* was used to tag bacterial inoculants. This transposon carries a kanamycin-resistance gene (*nptII*) under the control of a constitutively expressing promoter and it inserts stably into the chromosome of their hosts (Reznikoff 1993). Tagged strains could specifically be detected among naturally resistant soil bacteria

after growth on kanamycin-amended agar with a transposon-specific gene probe (Fredrickson et al. 1988; Pillai and Pepper 1991). Like Tn*7*, the transposon Tn*5* has a very broad host range, which allows the application of this tool to a wide variety of biotechnologically important species, Tn*5* even including the Gram-positive Bacilli (Obukowicz et al. 1986). In contrast to Tn*7*, Tn*5* has the potential to integrate into many different sites of a chromosome, which consequently generates a large diversity of genotypes from a single host organism (de Bruijn 1987). Theoretically, the tagged host organisms are not "genetically engineered" if an unmodified Tn*5* is delivered into the chromosome. However, it has been noted that in the process of constructing Tn*5* strains, sequences of the transposon delivery vector may also integrate into the chromosome (Fredrickson et al. 1988). As most vectors are in fact genetically engineered for an optimised performance in the laboratory, hosts with so-called vector integrates are also genetically engineered. The identity of transposon-tagged cells can be confirmed by gene probe hybridisation with grown colonies (Fredrickson et al. 1988), or by polymerase chain reaction (PCR), directly extracted from inoculated soil (Cullen et al. 1998).

Transposons themselves, once inserted into a chromosome, do not lose their potential to further move on to other genetic entities, e.g., to conjugative plasmids and to other hosts (Olson et al. 1991; Natarajan and Oriel 1992; Mahillon 1998). This is also the case for other transposons, such as the previously mentioned Tn*7*. In contrast to Tn*5*, Tn*7* has a smaller host range and fewer chromosomal sites at which it may integrate (Staley et al. 1997). Depending on the objective of modifying or tagging an inoculant, a certain transposon and delivery system can be advantageous. Since the development of these first constructs in the second half of the 1980s, several other genetic systems have been applied to tag a large diversity of prospective soil inoculants, and transposon delivery vectors have been optimised as "miniTn*5*", "miniTn*7*", or "miniTn*10*" to become non-transposable, once inserted in the chromosome (Ely 1985; Simon et al. 1989; Furukawa et al. 1991; Kaniga and Davison 1991; Alexeyev et al. 1995; Fedi et al. 1996; de Lorenzo et al. 1998; Xi et al. 1999; Lambertsen et al. 2004).

Alternatively to transposons, site-specific homologue recombination can also be used to anchor recombinant marker genes in specific positions of the chromosome (Bailey et al. 1995; Court et al. 2002; Wendland 2003). It is likely that this latter technique will become increasingly important as our knowledge about microbial genome sequences increases. Concomitantly to the development of more genetic tools for gene insertions, further selective techniques and detection markers have become available to construct microbial inoculants designed for environmental release (Prosser 1994; Jansson 1995, 2003; Wilson 1995; Jansson et al. 2000).

18.3
Selective Markers

The selection process is an important means to separate the tagged strains from the normally much higher populations of indigenous microorganisms. Antibiotic resistances combined with a cultivation on selective agar amended with the respective antibiotics are still one of the major and most efficient tools to achieve this goal (Halverson et al. 1993; Van Elsas et al. 1998; Corich et al. 2001; Amarger 2002). As all antibiotics have specific host ranges, certain microbial groups can be completely inhibited while others remain more or less unaffected. Consequently, to inhibit growth of as many non-target microorganisms as possible, a selection with two or three combined antibiotics may be advisable. This approach then requires that the selected strain for monitoring is resistant to all of these compounds. Resistances of a strain to be monitored can be selected by picking spontaneous resistant mutants or by serial adaptations of the strains to increasing amounts of the antibiotics. Alternatively, resistances can be inserted by genetic engineering. Examples for spontaneous or selected resistances for soil bacteria such as *Pseudomonas* spp. or *Rhizobium* are those against rifampicin and streptomycin (Dammann-Kalinowski et al. 1996; Han et al. 2003; Lilley et al. 2003), whereas resistances for these species against kanamycin or tetracycline can conveniently be achieved by transfer of the respective resistance genes *nptII* or *tet*, e.g. located on a transposon or plasmid.

Due to the tremendous importance of antibiotics for human health and the risk of horizontal gene transfer from a potential inoculant to health-relevant microorganisms, it must be made very clear that only resistance genes of clinically unimportant antibiotics should be considered as selective markers and a general abstention from using antibiotic genes in such a context has been discussed, as there are ample alternatives (Jansson 1995). Such alternative markers to select specific organisms against the high background of soil bacteria are resistances against heavy metals such as mercury or nickel, or against the half-metal tellurite. As for antibiotics, the resistances can either be spontaneous or mediated by resistance genes, i.e. by *merA* (Barkay et al. 2003), *nre* (Stoppel and Schlegel 1995), or *telA*/*telB* (Taylor 1999). Tellurite resistance may be an especially interesting alternative to antibiotic resistances, because its toxicity as supplied to growth media is relatively low and so is the background of indigenous resistance in the soil microbial community (Kinkle et al. 1994; Sanchez-Romero et al. 1998; Mougel et al. 2001; Zanaroli et al. 2002).

A further option to avoid the use of antibiotic resistances as selective markers is to select inoculants that can grow on unusual substrates, i.e. a C source that cannot, or can only rarely, be degraded by members of

the indigenous soil microbial community. These metabolic markers can be used for selection, for specific detection, or for both, as described earlier for the *Pseudomonas* spp. carrying *lacZY* genes for degrading and growing on lactose. The *lacZ* marker coding for β-galactosidase can also be used only for detection as *lacZ*-expressing colonies on agar can be visualised with X-Gal. *Rhizobium leguminosarum* tagged with a *lacZ* gene has been used in field trials in Italy (Corich et al. 1996). Similarly, other soil bacteria, among them several members of the *Rhizobium* group, have been constructed to express a β-glucuronidase encoded by *gusA* or a β-glucosidase encoded by *celB* (Selbitschka et al. 1992; Sessitsch et al. 1996, 1998; Xi et al. 1999). As described for *lacZ*, the activities of these hydrolases can be detected with specific substrate conjugates, i.e. X-GlcA (5-bromo-4-chloro-3-indolylglucuronide). The background of indigenous bacteria cleaving X-GlcA from soil, however, can be relatively high if cultivations are conducted on a non-selective agar, but, combined with a kanamycin-resistant marker, no background was detected in a loamy soil (Wilson et al. 1994). This marker is also highly useful to directly detect tagged rhizobial inoculants in the nitrogen-fixing root nodules of legumes caused by infections of the inoculants (Wilson et al. 1995). *Sinorhizobium meliloti* tagged with a *gusA* gene has been released in field studies in Spain (Van Dillewijn et al. 2001).

The *xylS* gene codes for an activator protein which confers the capacity to grow on methyl benzoate as a sole source of C. The marker has been used in combination with the kanamycin-resistance gene of miniTn*5* to follow the fate of a *P. putida* strain in field studies in soil and rhizospheres and to test a containment of the inoculated cells by induction of a suicide pathway in the absence of methyl benzoate (Molina et al. 1998). Another gene of the same operon is the *xylE* coding for the 2,3-catechol dioxygenase. This gene has been used in a number of *Pseudomonas* strains that were inoculated into soil microcosms (Macnaughton et al. 1992), on plant surfaces in greenhouse studies (de Leij et al. 1994; Thompson et al. 1995a), and on plants in the field (de Leij et al. 1995a; Thompson et al. 1995b). An interesting marker is the *moc* region of *Rhizobium radiobacter* (formerly: *Agrobacterium tumefaciens*), coding for manitol opine catabolism, which has been transferred to a *P. fluorescens*, allowing a sensitive detection of that strain even after non-selective cultivation from inoculated soil samples (Hwang and Farrand 1994, 1997).

18.4 Luminescence and Fluorescence Markers

Luminescence and fluorescence markers are highly specific with little or no background in the indigenous soil microbial community. Their importance

as in situ biosensors has been discussed in Chaps. 16 and 17 of this volume. The markers are ideal not only in regard to their specificity, but also because the detection of luminescence or fluorescence is straightforward. However, for monitoring in the field, luminescence and fluorescence markers normally need to be combined with other selectable markers as they themselves have no directly selectable properties. Luminescence markers convey the capacity to produce light. Two types of genes can provide this phenotype, i.e. the bacterial luciferase of marine bacteria such as *Vibrio fischeri* or *V. harveyi*, and the eukaryotic firefly luciferase (Prosser 1994; Prosser et al. 2000). The bacterial luciferase consists of two subunits which are encoded by the *luxAB* genes. The enzyme catalyses the oxidation of $FMNH_2$ and decanal with molecular oxygen under a concomitant light emission. The natural host bacteria are autobioluminescent, as they synthesise the substrate decanal themselves. The genes for this biosynthetic pathway are located in the same genetic unit (operon) as the luciferase genes (*luxCDABE*).

For monitoring purposes, bacterial inoculants can be prepared with either the luciferase alone or with the complete *lux* operon. In the first case, luminescence production depends on the addition of decanal, e.g. onto colonies grown on agar, in the second case, the cells become autoluminescent, provided that the complete operon is translated into functioning proteins. It is unlikely that field inoculants will be designed with the latter option, because autoluminescence is a metabolic burden which may debilitate the host, as it is not needed in soil. In contrast, *luxAB* provides an interesting means of tagging an organism, as decanal is an inexpensive substrate which can be added to colonies re-isolated from soils but also directly to cells, e.g. as they are attached to a root surface (rhizoplane). The direct detection can be achieved by incubating the biological material after decanal addition with a light-sensitive film. The expression of *luxAB*-encoded bioluminescence has also been used to detect metabolically active bacterial cells on rhizoplanes in order to evaluate the performance of potential biocontrol strains (de Weger et al. 1991, 1995; Rattray et al. 1995; Schmidt et al. 1997; Dunne et al. 2000; Ma et al. 2001). It has also been suggested as a suitable marker to tag a plant growth promoting *P. putida* strain for a deliberate field release (Ramos et al. 2000).

The use of *luxAB*-marked inoculants can further be extended by combining them with a fluorescence marker such as *gfp*, which codes for the green fluorescent protein (GFP), as this combination allows the study of both the abundance and the activity of inoculated bacteria simultaneously, e.g. in rhizoplanes (Unge et al. 1999; Unge and Jansson 2001). In contrast to the luciferase encoded by *luxAB*, the GFP is a non-catalytic protein. To detect GFP, the amount of protein in a cell, however, must be relatively high compared to a catalytic activity, such as luciferase. Such a high content of

a foreign protein might interfere with the fitness of an inoculant and, thus, GFP may not be a good tag for following bacteria in the context of a field release. On the other hand, as GFP can be detected independently of cultivation, the problem of non-culturability can be circumvented. Unstable variants of GFP can be used to directly detect and quantify in situ gene expression (Andersen et al. 1998). Another interesting alternative is to use GFP-tagged cells for cultivation-independent enrichment by flow cytometry (Tombolini et al. 1997), even though this latter approach is probably not directly applicable to soil due to interference with soil particles. Numerous examples demonstrate the usefulness of GFP tagging of inoculants with a biocontrol potential to study their survival and interaction with different crops (Bloemberg et al. 1997; Unge et al. 1998; Adilova et al. 2001; Lee et al. 2002; Zhu et al. 2002; Gage 2004).

The eukaryotic luciferase, encoded by the *luc* gene, catalyses oxidation of D-luciferin, a heterocyclic carboxylic acid, with molecular oxygen to oxyluciferin under the hydrolysis of ATP to AMP, and generates carbon dioxide and light. The quantum yield of the reaction is much higher than for the bacterial luciferases and details of the system have been described elsewhere (Prosser et al. 1996). The *luc* genes have been optimised to function in bacterial cells and cells equipped with these genes have the potential to emit light after addition of luciferin (Cebolla et al. 1995). The emission can be detected from colonies grown on agar after blotting of the cells onto nylon membranes which are sprayed with luciferin and then incubated with a light-sensitive film (Cresswell et al. 1994; Schwieger and Tebbe 2000; Fig. 18.1). Alternatively, bioluminescent cells can also be detected by photon imaging using cooled charge coupled device (CCD) technology, and suspended bioluminescent cells can also directly be detected by lumino-

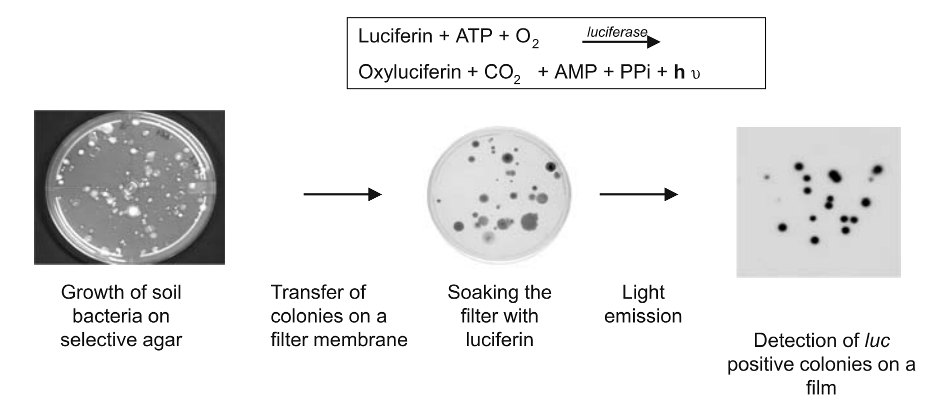


Fig. 18.1. Cultivation-dependent detection of luciferase-tagged bacterial cells within the soil microbial community

metry (Prosser et al. 1996, 2000). This is especially attractive for the analyses of nodulating Rhizobia, as the presence of the luciferase can directly be detected with crushed root nodules in a luminometer (Miethling and Tebbe 2004). To allow selection of *luc*-tagged cells, several different resistances have been used, e.g. spontaneous, chromosomally located resistance against streptomycin (Dammann-Kalinowski et al. 1996) or chromosomal insertion of a combined *luc-mer* cassette to confer a selectable mercury (Hg^{2+}) resistance (Björklöf and Jørgensen 2001). The *luc* gene has been inserted into the chromosome of several bacterial species that were designed for field releases, and we give the example for using *luc* as a marker in field releases later.

18.5 Objectives for Field Releases of Genetically Engineered Bacteria

Motivations for conducting experimental field releases with genetically engineered bacteria can be merely scientific, e.g. to understand general patterns of how introduced organisms survive and interact with their environment, but they can also be conducted for an applied purpose, e.g. to evaluate the performance of a potentially commercial product for agriculture. Examples for field studies with marker gene-tagged bacteria are shown in Table 18.1. From today's perspective, the promise of successful use of bacterial inoculants in an agricultural context seems higher than that of using inoculants to clean-up polluted soils (Ripp et al. 2000; Amarger 2002). Most deliberate field releases have so far been conducted with bacteria that either enhance crop nutrition or that can act as biocontrol agents. Among the inoculants that enhance plant nutrition, two groups can be distinguished, i.e. the group of Rhizobia which lives in symbiosis with associative leguminous crops (Amarger 2001; Sessitsch et al. 2002) and the group of asymbiontic plant growth promoting rhizobacteria (Lucy et al. 2004).

Field studies with marker gene-tagged Rhizobia have been conducted to analyse competition between inoculated cells and indigenous nodulating bacteria, to determine the risk of horizontal gene transfer, to enhance nodulation and nitrogen fixation by genetically optimised strains, or to study the efficiencies of biological containment systems. As interactions between Rhizobia and their host plants are specific, different species have been released in context with different leguminous host plants, e.g. *Rhizobium leguminosarum* for nodulating pea (Corich et al. 1996; Hirsch 1996), *S. meliloti* for alfalfa (Bosworth et al. 1994; Scupham et al. 1996; Tebbe 2000), *R. etli* for beans (*Phaseolus vulgaris*; Robleto et al. 1997), or *Bradyrhizobium japon-*

Table 18.1. Examples of genetically engineered bacterial inoculants that have been used in field release studies

Species	Objectives	Markers[a]	Reference
Azospirillum brasilense	Evaluation of the marker gene for field monitoring and detection of ecological impact	*lacZ* or *gusA*	Basaglia et al. 2003; Nuti et al. 2003
Pseudomonas aureofaciens	Validation of microcosms to predict field survival	*lacZY*, Rif^r, Nal^r	Angle et al. 1995
Pseudomonas fluorescens	Impact on indigenous soil bacteria in the rhizosphere	*lacZY*, Kan^r, *xylE*	de Leij et al. 1995a,b
P. fluorescens	Biocontrol of rhizomania by increased production of antimicrobial metabolite	*gusA*, Rif^r	Resca et al. 2001
Pseudomonas putida	Efficiency of a suicide containment system for a pesticide-degrading inoculum	*xylS*, Kan^r, growth on 3-methylbenzoate	Molina et al. 1998
P. putida	Efficiency of a biocontrol agent with antifungal activities and detection of side effects on indigenous soil microorganisms	Rif^r, Kan^r	Glandorf et al. 2001; Viebahn et al. 2003
Rhizobium leguminosarum bv. *viciae*	Survival in soil, efficiency of the gusA marker, detection of horizontal gene transfer	*gusA*, Str^r	Selbitschka et al. 1995b; Hirsch 1996
R. leguminosarum bv. *viciae*	Marker stability, competition with indigenous bacteria, effect on soil microorganisms	Rif^r, Hg^r, *lacZ*	Corich et al. 2000
Sinorhizobium meliloti	Improvement of N fixation and growth of alfalfa by increased expression of the nitrogenase operon or improved C source supply of bacteroids in root nodules	Str^r, some with Kan^r	Bosworth et al. 1994; Donegan et al. 1999
S. meliloti	Improvement of nodulation efficiencies by a modified proline-dehydrogenase	Str^r, Hg^r	van Dillewijn et al. 2001, 2002
S. meliloti	Efficiency of luc marker for field monitoring, test of *recA*-mutant for biological containment, effect on soil properties	*luc*, Str^r	Schwieger and Tebbe 2000; Schwieger et al. 2000; Tebbe 2000; Selbitschka et al. 2003

[a] Hg^r, Kan^r, Nal^r, Rif^r, Str^r: resistances against mercury (Hg^{2+}), kanamycin, nalidixic acid, rifampicin or streptomycin; Metabolic markers: expression of β-galactosidase (lacZ), or β-galactosidase and lactose permease (lacZY; lactose utilisation), β-glucoronidase (*gusA*), 2,3-catechol dioxygenase (xylE), or activator protein of the xyl-operon (*xylS*)

icum for soybeans (cited in Amarger 2002). Several genetically engineered strains with potentially improved properties in regard to their nodulation or nitrogen fixation have recently been constructed, but have, to our knowledge, not yet been tested in the field (Castillo et al. 1999; Soberon et al. 1999; Bascones et al. 2000; Marroqui et al. 2001). Examples for asymbiotic plant growth promoting rhizobacteria that have been studied in the field are strains of *P. fluorescens* (de Leij et al. 1995a,b; Thompson et al. 1995b) and other *Pseudomonas* spp. (Choi et al. 2003; Viebahn et al. 2003), as well as different strains of *Azospirillum brasilense* (Basaglia et al. 2003).

The root-colonising potentials of the asymbiotic rhizobacteria have also been used to deliver insecticidal crystal proteins of *Bacillus thuringiensis* (Bt-toxins; Schnepf et al. 1998) to the root surface and thereby protect plants against soil-borne phytopathogens. The host change from the natural host to such bacteria is expected to increase the concentrations of these insecticidal agents in the rhizospheres, as these are the "hot spots" where the insecticidal activity is actually needed (Udayasuriyan et al. 1995; Kaur 2000; Peng et al. 2003). However, there is a lack of information about the field performance of genetically engineered bacterial inoculants with Bt-toxins and it is still difficult to predict whether such products will become competitive to the now widely used transgenic crops expressing Bt-toxins (Clark and Lehman 2001). In fact, only a few genetically engineered inoculants have already been commercialised, e.g. some *Rhizobium* strains with an improved nodulation or nitrogen fixation capacity for application in the US (Bosworth et al. 1994) and an *R. radiobacter* (syn. *A. tumefaciens*) to control crown gall disease caused by wild types of this species in Australia (Ryder 1994).

In the following sections, we will report results from a field study that was conducted in order to determine the field performance of *S. meliloti* strains as inoculants for alfalfa. The major motivation of this field study, which included the first deliberate field releases of genetically engineered bacteria in Germany, was to determine the efficiency of the *luc*-marker gene for field monitoring purposes, to characterise patterns of survival in soils with different histories of agricultural use, and to test a *recA*-mutation as a means of biological containment of genetically modified microorganisms.

18.6 Field Release of *Sinorhizobium meliloti* L33 and L1 – A Case Study

Two *S. meliloti* strains were selected for field releases in conjunction with their host for forming root nodules, alfalfa. Both strains were derived from the same parental strain, *S. meliloti* 2011, a non-engineered isolate with

a spontaneous streptomycin resistance (Selbitschka et al. 1992; Dammann-Kalinowski et al. 1996). The selected strains both carried a chromosomal insertion of the *luc* gene connected to a constitutively expressing *nptII* promoter. In strain L33, this marker gene expression cassette was inserted downstream of the *recA* gene with no significant effect on the phenotype, except for mediating bioluminescence after the addition of the substrate luciferin. In strain L1, however, the cassette was inserted *into* the *recA* gene, resulting in a mutant which lost the capacity to produce a functioning RecA protein. The RecA protein is involved in homologue recombination and DNA repair (Takahashi et al. 1996). Laboratory and small-scale microcosm experiments with the RecA-deficient strain *S. meliloti* L1 showed decreased growth rates, reduced survival in soil under stress conditions, and reduced nodulation competitiveness compared to its counterpart *S. meliloti* L33 (Dammann-Kalinowski et al. 1996; Hagen et al. 1997; Niemann et al. 1997; Dresing et al. 1998). These properties confirmed the concept of using a *recA*-mutant of *S. meliloti* as a possible means of biological containment under field conditions (Selbitschka et al. 1992, 1995a). However, greenhouse studies with lysimeters filled with soil from a prospective field release site, cropped with alfalfa, did not reveal such differences (Schwieger et al. 1997). In studies with a *recA*-mutant of another *S. meliloti*, no differences to the wild type were found in the survival of the inoculants in soil but differences were, in fact, detected in regard to a decreased symbiotic performance (Herrera Cervera et al. 1997).

The first field releases of *S. meliloti* L33 and L1 were conducted in September 1994 using field lysimeters which had been established on an agricultural field at the Federal Research Centre for Agriculture (FAL) in Braunschweig, Lower-Saxony, Germany. The field lysimeters had the same dimensions as those that had been used in the greenhouse, i.e. a 60 cm soil profile with a diameter of 31.5 cm. The lysimeters were inoculated with either one of the two *luc*-tagged strains or with the wild-type strain, *S. meliloti* 2011 (Schwieger et al. 2000; Tebbe 2000). For each treatment and non-inoculated controls, four replicate lysimeters were included in that study. The lysimeters were inoculated with peat-soil-cell mixtures prepared in the laboratory to reach an initial concentration of approx. 10^6 cells g^{-1} soil in the upper 20 cm soil horizon. The cell concentrations were determined by cultivation on selective agar as colony-forming units (cfu) and luminescence was detected by adding the substrate luciferin.

The survival, vertical dissemination and the effect of the inoculants onto soil chemical and microbial parameters were monitored over a period of 2 years. A typical initial decline in cell numbers of both *luc*-tagged strains was observed during the first months in winter reaching a concentration of approx. 10^4 cfu g^{-1}. At this level, the populations were maintained for 2 years until the plants were removed. During the presence of alfalfa, signif-

icant differences in cell numbers of the two isogenic strains L33 and L1 were not detected, except for a single observation, exactly 1 year after inoculation, at which the titre of L33 was significantly higher than that of L1. After removal of the host plants from the lysimeters in spring of the second year, the titres of both strains dropped within 6 weeks by 0.5 orders of magnitude, but *S. meliloti* L33 populations then remained stable, whereas the L1 populations further declined to 3×10^3 cfu g^{-1} soil. Thus, confirmed by two events at which significant differences were detected, there were clear indications that the *recA* mutation, in fact, altered the ecological behaviour of *S. meliloti*.

A transport of the cells into deeper soil layers or even further down through the whole 60 cm depths of the lysimeter soil was not detected throughout the 2 years, even though each lysimeter received an average of 42.5 L of naturally precipitated rain water. The analyses of root nodules indicated that the inoculants strains stayed in their lysimeters, except for a single observation in which a bioluminescent root nodule was found in an alfalfa plant grown in soil taken from a non-inoculated control lysimeter (Schwieger et al. 2000). None of the inoculations caused any growth response by alfalfa.

The second and third field releases of these strains were conducted on field plots at two sites, one in Braunschweig, next to the lysimeters, and one in Strassmoos, Bavaria, southern Germany. The soils at both sites were relatively similar in regard to their structure and many chemical properties but the history of agricultural use was different. In Braunschweig, alfalfa had not been grown during the previous decade but it was commonly grown at the Strassmoos site. It is known from a number of studies that the presence of alfalfa stimulates the growth of *S. meliloti* (Bromfield et al. 2001; Da and Deng 2003) and, in fact, field site monitoring confirmed that the populations of indigenous *S. meliloti* were much higher in Strassmoos than in Braunschweig. The field plots in Braunschweig were inoculated in April 1995, the field plots in Strassmoos in August 1997. As already observed with the lysimeter studies, none of the inoculations at either site in fact stimulated the growth of alfalfa. In both studies, the NO_3 concentrations of soil were so high that available N did not limit plant growth. In other studies with soil from the same site but with very low N concentrations, inoculation effects on plant growth could clearly be seen.

In Braunschweig, both *S. meliloti* L33 and L1 as well as *S. meliloti* 2011 were each released onto five 9 m^2 field plots that were arranged in a randomised order. The plots were separated by grass sections of 3 m width. At the time of release, the level of indigenous *S. meliloti* was below the threshold of detection, which in this case corresponded to 100 cfu g^{-1} soil. All plots had been seeded with alfalfa 1 day before the inoculation. The plots were inoculated by spraying bacterial cell suspensions onto the soil surface, using a device that is normally used for accurate and targeted field applications of

pesticides. The objective was to reach an initial inoculant concentration of 10^6 cells g^{-1} soil in the plough horizon. The analysis of the aerial drift of the bacteria during the inoculation process resulted in a spread of 2 cfu cm^{-2} on average at a maximal wind velocity of 3 m s^{-1} (Tebbe 2000; Selbitschka et al. 2003). As already observed in the field lysimeter study, the inoculants decreased during the first 3 months by about two orders of magnitude, i.e. from 1×10^6 cfu g^{-1} to 3×10^4 cfu g^{-1}. Later on, however, both *S. meliloti* L1 and L33 persisted at a population level of $1.98\pm1.70\times10^4$ cfu g^{-1} for several years on the field plots with alfalfa, with no significant differences (Fig. 18.2a). In April 1999, 4 years after the release, the old alfalfa plants, which no longer homogenously covered the plots, were removed and alfalfa was seeded again. This activity, however, did not stimulate the populations of *S. meliloti* L33 or L1 but in contrast resulted in a further decline to approx. 10^2 cfu g^{-1} in April 2002. This effect was probably caused by the emergence of an indigenous *S. meliloti* population which could be detected in nodulation assays (see below).

A horizontal spread of the inoculants was observed at the field site, but the success of this spread was clearly linked to the presence of alfalfa. It was possible to detect *luc*-positive cells below a titre of 10 cfu g^{-1} soil immediately after the inoculation procedure, but all subsequent samplings were negative. In contrast, 12 weeks after inoculation, *luc*-positive *S. meliloti* cells were detected in the rhizospheres of alfalfa which had grown on the non-inoculated control plots. Two weeks later, an average titre of 22 cfu g^{-1} soil was determined, which increased further to concentrations as seen on the inoculated plots (Schwieger and Tebbe 2000). Interestingly, the strain that was detected on these non-inoculated plots was exclusively *S. meliloti* L33, which was caused by the absence of indigenous *S. meliloti* and the weak ability of the RecA-deficient *S. meliloti* L1 to compete against strain L33.

In Strassmoos, the field site was inoculated with a sand-peat mixture, with the *S. meliloti* cells pre-immobilised in the peat fraction. As in the other experiments, the initial concentration of the inoculant in the plough horizon was 10^6 cells g^{-1}. The size of each single field plot was similar to the Braunschweig site (10 m^2). Crop rotation was included as another treatment at Strassmoos. For biometric reasons, the number of replicates was higher than at the FAL site, with 14 plots inoculated with *S. meliloti* L33, 14 plots with *S. meliloti* L1, and 7 plots with *S. meliloti* 2011. As in Braunschweig, each plot was bordered by grass. When the plots were inoculated, the level of indigenous *S. meliloti* in the field soil was approx. 10^5 cells g^{-1}. Compared to the liquid inoculation technique applied in Braunschweig, this improved inoculation procedure reduced the aerial drift of the inoculated bacteria by more than 90% to 0.16 cells cm^{-2} with a maximal wind velocity similar to Braunschweig (2.5 m s^{-1}). Already 5 weeks after inoculation, the titres of

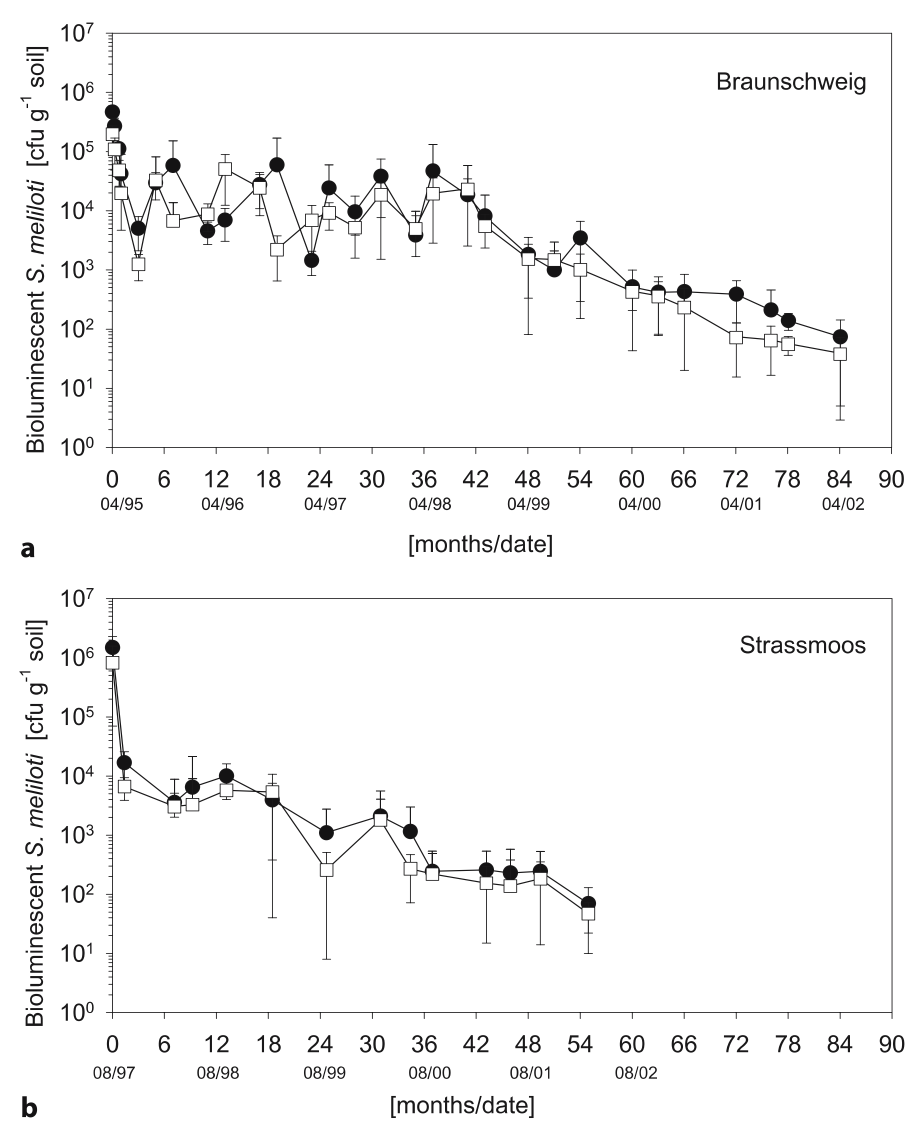


Fig. 18.2. Survival of luciferase-tagged *Sinorhizobium meliloti* strains L33 (RecA$^+$; ●) and L1 (RecA$^-$; □) on field plots in Braunschweig (**a**) and Strassmoos (**b**). Each value represents mean and standard deviations of cell numbers from five (Braunschweig) and seven (Strassmoos) replicate field plots, respectively

both *S. meliloti* strains had declined to 9×10^3 cfu g^{-1} and further on they maintained an average population size of 6.6×10^3 cfu g^{-1} for 2 years on the field plots with alfalfa with no significant deviation due to their different *recA* genotype (Fig. 18.2b). In the following years the concentrations de-

creased continuously to a final level of 60 cfu g^{-1} in March 2002, when the field study was finished.

The competition between the inoculants and the indigenous *S. meliloti* populations was studied by nodulation assays, i.e. in which soil from the field sites was incubated in laboratory microcosms with alfalfa seeds. Colonisation of the developing root nodules, which are normally caused by single *S. meliloti* infections and therefore clonal, was studied by luciferase detection and genetic fingerprinting. At the Braunschweig site, with no initial indigenous *S. meliloti* population, the nodule occupancy was dominated by the inoculants. In 1995 and 1996, more than 90% of the nodules were infected by the bioluminescent strains with no cross-contaminations between the different plots. However, already 6 months after the release, indigenous *S. meliloti* strains were also detected. In 1997 and 1998, the proportion of these soil-borne *S. meliloti* increased to 35% on plots inoculated with *S. meliloti* L33 and even 65% on plots inoculated with *S. meliloti* L1. After reseeding of the plots in 1999, the nodule occupancy of the indigenous *S. meliloti* increased further and *S. meliloti* L1 and L33 were only detected in 2% and 10% of the nodules, respectively (Selbitschka et al. 2003). In July 2000, the concentration of the total nodulating *S. meliloti* population was 10^4 cells g^{-1} soil, as determined by most probable number analyses, representing 96% indigenous *S. meliloti* on average. At the Strassmoos site, during the first year after inoculation, more than 90% of the nodules were infected by the *luc*-tagged strains but this proportion decreased dramatically in the following year to less than 10% of the nodules being colonised by *luc*-positive cells. In 1999 and 2000, the infection rate decreased further and dropped below 5%, confirming that the selective force of the indigenous *S. meliloti* populations correlated with their population size, which was lower in the Braunschweig soil (Selbitschka et al. 2003).

In addition to the data presented here, a large amount of work was dedicated to detect non-deliberate effects, i.e. horizontal gene transfer of the marker gene to indigenous *S. meliloti* and other bacteria and changes in the abiotic and biotic properties of the soils caused by the presence of the genetically engineered strains. The results have been presented in detail elsewhere (Schwieger and Tebbe 2000; Schwieger et al. 2000; Tebbe 2000; Selbitschka et al. 2003). Despite the use of a highly specific assay to detect horizontal gene transfer by analyzing *luc*-positive root nodules with genetic fingerprinting (Selbitschka et al. 2003), it was not possible to detect a single gene-transfer event. Thus, the *luc* gene was efficiently "locked" in its host strains. Even though the *luc*-tagged populations established themselves for a considerable time in the field soil, their population sizes, with e.g. 10^3 cells g^{-1} soil, were very small compared to the total number of bacterial cells which can be estimated with 10^8 to 10^9 cells g^{-1}. Thus, it was not surprising that none of the "classical" soil parameters, e.g. total C or

N, total microbial biomass, or also community-level-physiological profiles, were affected in any of the field studies with *S. meliloti* L33 or L1.

18.7 Evaluation of Strategies To Eliminate *S. meliloti* from Soil

The long-term survival of *S. meliloti* at the field site in Braunschweig, with approx. 10^3 cells g^{-1} soil, triggered the question how these populations could possibly be eliminated from soil. In order to evaluate a number of different strategies for this purpose, a greenhouse experiment was conducted. Six different strategies were compared with each other, using microcosms with soils that contained *S. meliloti* L33 populations established at 10^4 cfu g^{-1} in the presence of alfalfa: (1) crop rotation to clover and (2) rye, inoculation with two different competitor strains, i.e. (3) *S. meliloti* 2011 or (4) *S. meliloti* F16, the latter a strain previously isolated from the exact field site, and the application of (5) the herbicide Roundup (active ingredient: glyphosate) or the heavy metal copper (Miethling and Tebbe 2004). In bulk soil, only the application of Roundup, followed by tillage and subsequent seeding of grass, resulted in a significant reduction of the established *S. meliloti* inoculant, although a complete elimination was not achieved, leaving a final concentration of 45 ± 17 cfu g^{-1} soil after 83 weeks. Compared to the untreated control, as judged by the titre of cells in bulk soil, all other treatments failed (Fig. 18.3).

The survival of the established *S. meliloti* L33 in the rhizosphere, however, was clearly stimulated by alfalfa. The numbers of *S. meliloti* L33 cells in the rhizospheres of the non-host plants rye, clover and grass were two orders of magnitude below that found with alfalfa. Possibly, the discrepancy of data found between bulk and rhizosphere soil can be explained by the relatively short monitoring period. In other field studies, it was found that a population of soil-inoculated *S. meliloti* decreased from about 10^5 to 10^2 nodulating cells g^{-1} soil within 4 years after the alfalfa was harvested and succeeded by cereals (Hirsch 1996). In our study, the analysis of the alfalfa nodule occupancy after the addition of inoculants revealed a partial replacement of *S. meliloti* L33 by the strain F16, but not by the parental strain 2011, indicating that cell numbers of the inoculants were not as important under these conditions as strain-specific nodulation efficiencies (Miethling and Tebbe 2004).

As a consequence of these results, it was decided to apply Roundup as a means to eliminate as many *luc*-tagged *S. meliloti* cells as possible from the soils at both field sites. In fact, the measures adopted resulted in a decrease below the threshold of detection (<10 cfu g^{-1} soil) on both sites in 2002. After these treatments, the field in Strassmoos was re-integrated into

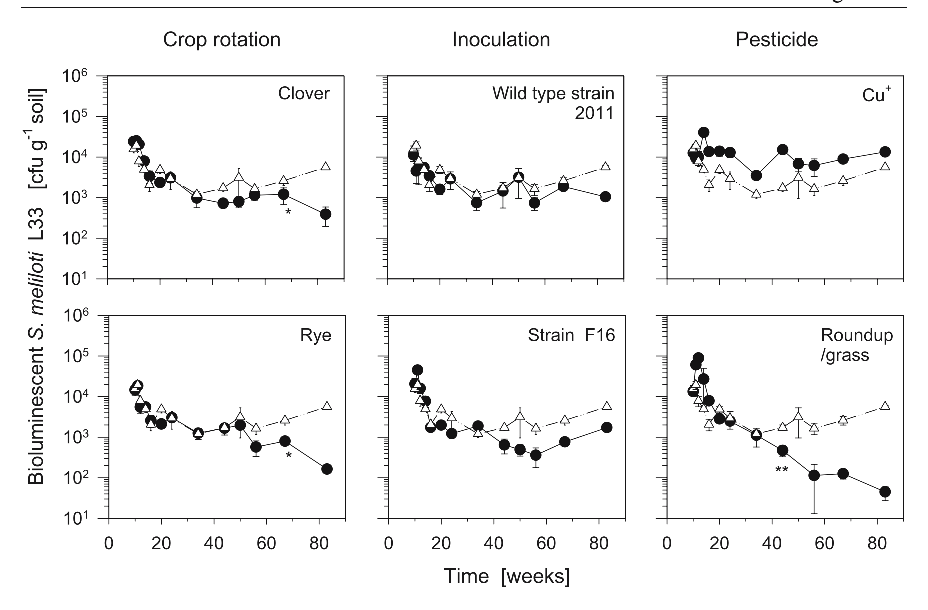


Fig. 18.3. Survival of a soil-established population of *S. meliloti* L33 in bulk soil in response to different soil treatments, i.e. crop rotation to non-host plants (clover and rye), inoculation with *S. meliloti* competitor strains at 10^6 cfu g^{-1} soil (wild-type strain 2011 and strain F16) and the application of pesticides [Funguran (Cu+) and Roundup]. Symbols: *S. meliloti* L33 in response to the respective treatments (•); *S. meliloti* L33 in control microcosms grown with alfalfa (△); (*) Total harvest of plants for rhizosphere investigation; (**) Seeding with grass (*L. multiflorum*). Values represent means and standard deviations of four replicate microcosms. Significance was tested using analysis of variance (ANOVA) at the 5% level (modified from Miethling and Tebbe 2004)

conventional agricultural management. At the site in Braunschweig, the detection of the *luc*-tagged strains goes on, approaching a decade of monitoring after their release. After a period of corn cultivation in 2003, the field site was again seeded with alfalfa in 2004, and further monitoring during the coming years will show whether the marker gene-tagged *S. meliloti* have in fact been eliminated.

18.8 Conclusions: Biosafety and Usefulness of Small-Scale Field Release Studies with Marker Gene-Tagged Bacteria

Despite the fact that the number of deliberate field releases of marker gene-tagged bacteria is still relatively low compared to field studies with genetically engineered plants, we can already look back over a significant amount of work which was conducted during the last 20 years in a number

of different countries and continents. Most of these studies were conducted just to follow the fate of inoculated cells into the environment and to test hypotheses about their behaviour as predicted by fine-scale molecular analyses and soil microcosm studies. Genetic engineering has been the crucial technique to equip bacterial inoculants with a selective and/or detection marker and thereby allowed the monitoring of their fate in the environment. Genetic engineering, however, is also the most powerful technology for improving inoculants, as it enables the development of much broader and more targeted solutions than classical mutation techniques. The discussion about the potential risks connected with the deliberate release of such optimised inoculants is as important as it was 20 years ago. The experiences made with the field releases thus far are one of the most important sources of information for discussion and risk assessment on a case-by-case basis. The most important message from these studies is that no such release has caused any known damage to human health or to ecosystem functions. On the one hand, this is not an encouragement to extrapolate a zero-risk expectation for the future. On the other hand, it is a clear indication of how valuable such small-scale field releases were and can be, to extend our knowledge about the interactions between microorganisms and their environment and to develop products that contribute to a sustainable use of biological resources in agriculture. It remains a challenge for environmental microbiologists not only to unravel and optimise the highly beneficial properties of microorganisms for environmental applications, but also to share this fascinating and important endeavour with the public.

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